

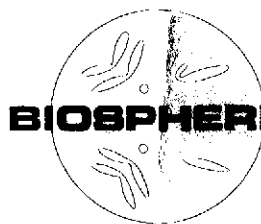
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"AUTOMATED MICROBIAL  
METABOLISM LABORATORY"

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APPENDIX I - Minutes - Mars Working Party  
of Immunological Studies Sub-Group, Held  
Friday, 30 November 1973

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I. SUMMARY

The design and rationale of an advanced labeled release experiment based on single addition of soil and multiple sequential additions of media into each of four test chambers are outlined. The feasibility for multiple addition tests has been established and various details of the methodology have been studied.

The four chamber battery of tests include:

- Chamber 1 - Determination of the effect of various atmospheric gases and selection of that gas which produces an optimum response.
- Chamber 2 - Determination of the effect of incubation temperature and selection of the optimum temperature for performing Martian biochemical tests.
- Chamber 3 - Nonbiological control for the other three test chambers. Sterile soil is dosed with a battery of  $^{14}\text{C}$  labeled substrates and subjected to the experimental temperature range.
- Chamber 4 - Determination of the possible inhibitory effects of water on Martian organisms is performed initially by dosing with 0.01 ml and 0.5 ml of medium, respectively. A series of specifically labeled substrates are then added to obtain patterns in metabolic  $^{14}\text{CO}_2$  evolution, which might provide evidence for the pathways of metabolism. The substrate series is followed by an anti-metabolite series designed to provide

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methods for microorganism control.  
(The temperature and atmospheric  
gas series should also provide infor-  
mation concerning methods of con-  
trol).

Thus far, the substrate series and temperature series have been performed fully in the multiple addition mode. This report includes data for the multiple addition substrate series, obtained with Meadow soil. Also reported herein, are the results of several investigations which led to the development of methodology for the substrate and temperature addition series.

A proposed method for the examination of nonsterilized or sterile samples of Martian soil based on immunological techniques seems promising. However, the problems associated with return of a nonsterilized soil sample from Mars have now been studied to the point where an administrative decision to proceed with conceptual and engineering design is required. The science community is not now in agreement about whether or not our present or near-term knowledge is sufficient to contain the possible risk such a sample might pose to the public health. The decision regarding the return of a nonsterile or a sterile sample will have a profound impact upon planning.

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II. EXPERIMENTAL TESTING

A. Multiple Addition vs. Single Addition:  
Temperature Effects

Experiments were performed to compare the  $^{14}\text{CO}_2$  evolution from a single medium addition with that from multiple addition. Triplicate vials containing 0.21cc of soil were dosed with 0.1 ml of VMI medium and incubated sequentially at 3°C, 20°C, 35°C and 60°C. Nine vials, each containing 0.21cc of soil, were also dosed with 0.1 ml of VMI medium and incubated at 20°C, 35°C and 60°C, in triplicate. These single addition vials were incubated for one hour at the experimental temperature prior to addition of the  $^{14}\text{C}$  labeled medium. Results are shown in Figures 1-3. At all three temperatures, the single addition mode produced lower peaks in activity and the time required for  $^{14}\text{CO}_2$  evolution to drop to baseline values was longer. The single addition produced two peaks in activity, the second peak being the greater. This two peak phenomenon was most pronounced at the 60°C incubation temperature. The multiple addition mode, on the other hand, produced a high single peak.

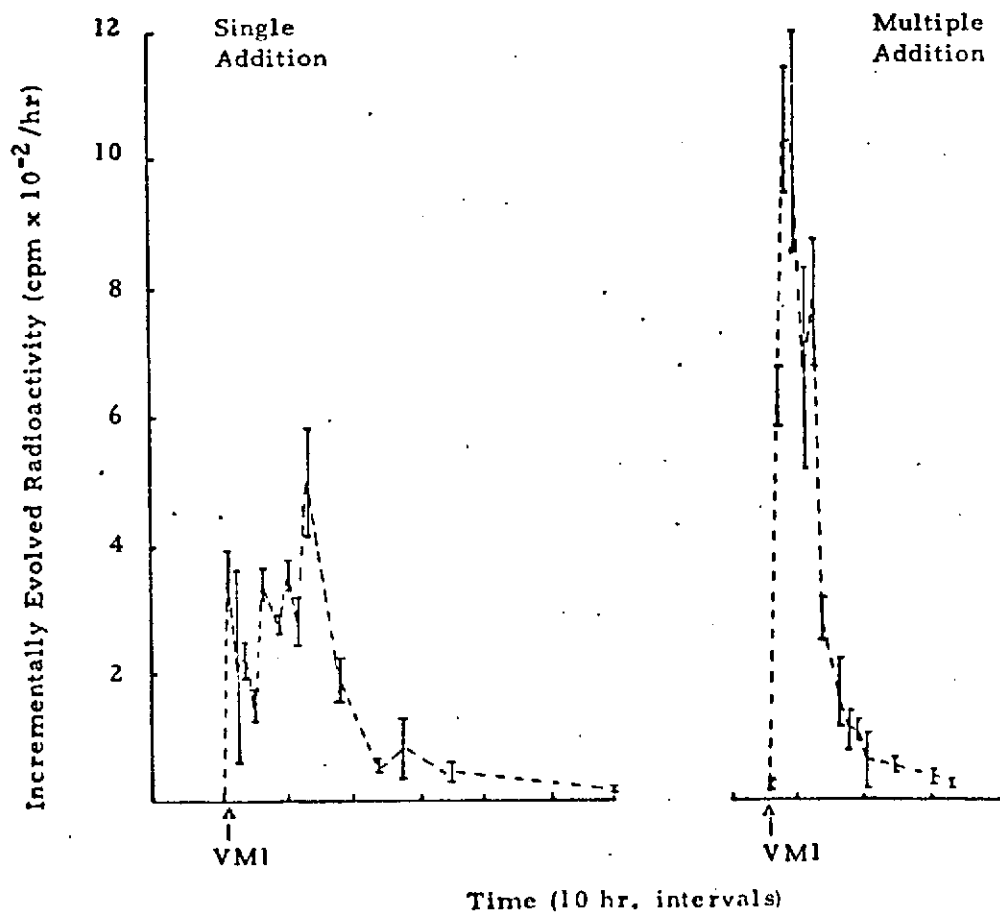
Both procedures, however, demonstrated that the Meadow soil was more active at 35°C and 60°C than at 20°C. The experiment

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Figure 1

Comparison of Multiple Addition  
and Single Addition Responses at 20°C

0.21cc meadow soil  
0.1ml VM1 medium ( $3.4 \mu\text{Ci/ml}$ )  
Data represents triplicate results

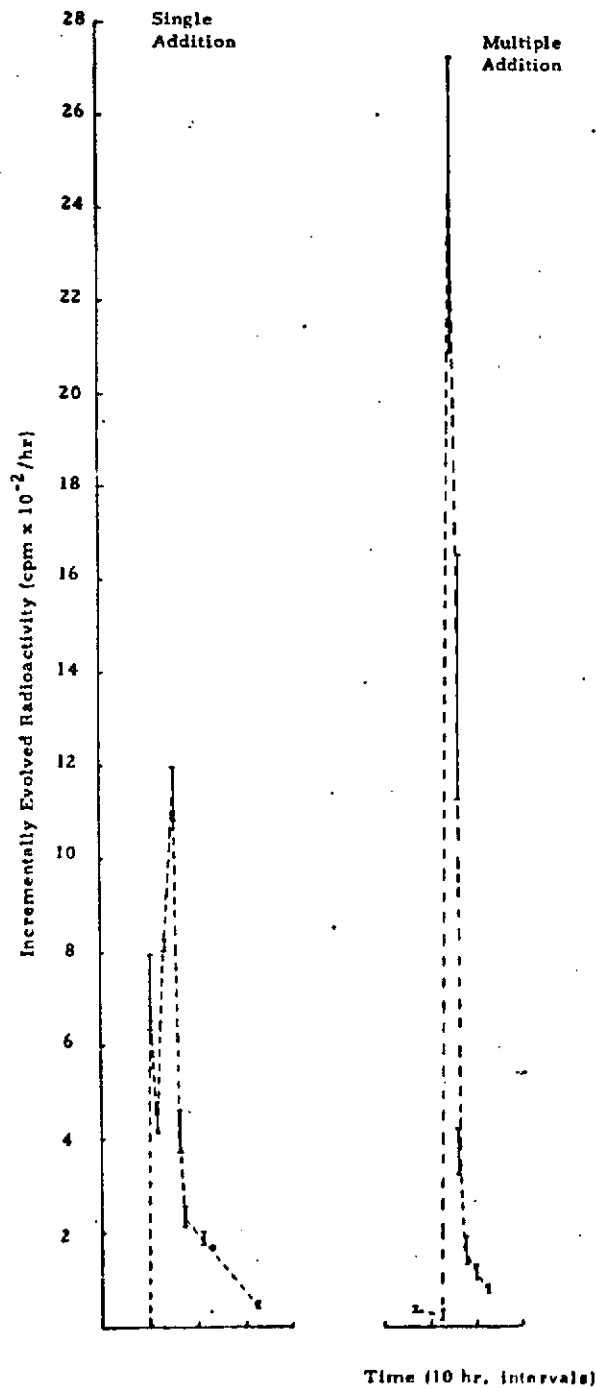


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Figure 2

Comparison of Multiple Addition  
and Single Addition Responses at 35°C

0.21cc Meadow Soil  
0.1ml VMI medium (3.4  $\mu$  Ci/ml)  
Data represents triplicate results

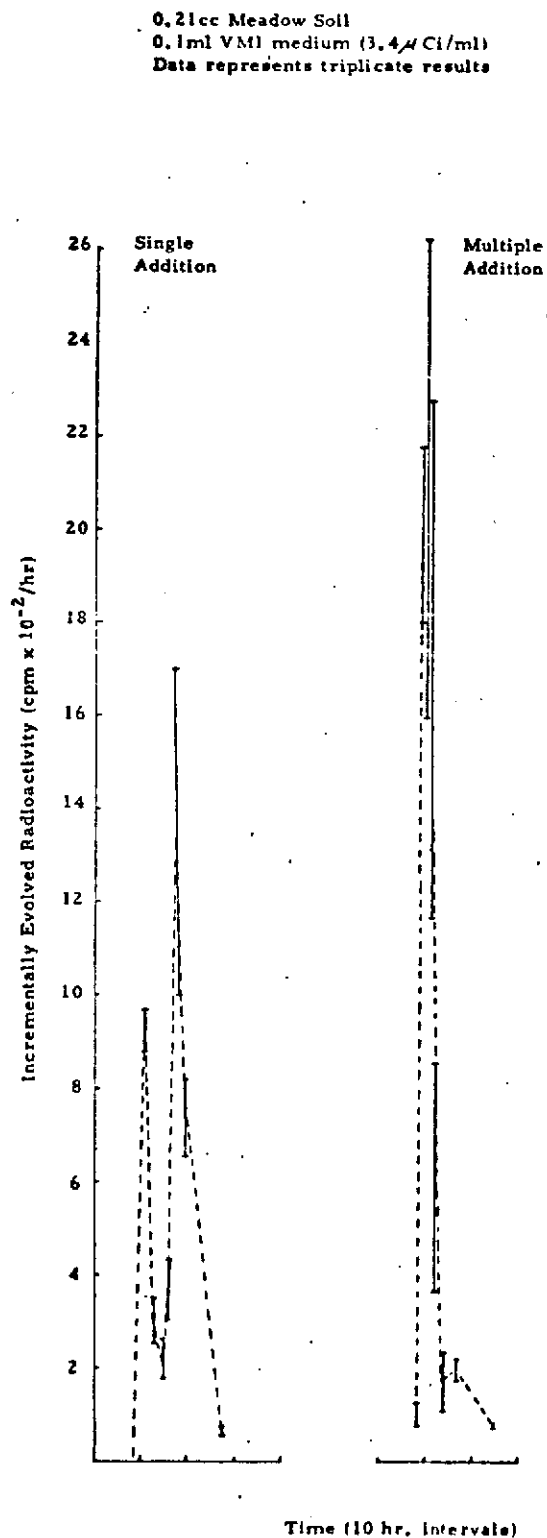




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Figure 3

Comparison of Multiple Addition  
and Single Addition Responses at 60°C



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shows that either the single addition or the multiple addition modes may be used to show the effect of temperature. However, comparisons between specific substrates or incubation conditions should exclude the comparison of first addition response to a subsequent addition response. This in no way invalidates the multiple addition mode of experimentation. But does require that comparisons of a series of additions exclude the first such addition.

### B. Specifically Labeled Lactate and Acetate: Single Addition

The possibility of using specifically labeled lactate and acetate to obtain comparative biochemical information was investigated experimentally. It had been found in earlier experiments that the patterns of  $^{14}\text{CO}_2$  evolution from  $^{14}\text{C}$  formate and from uniformly labeled lactate and acetate varied somewhat depending upon the soil. Lactate and acetate are closely related to pyruvate and acetyl-CoA, two key intermediates in terrestrial metabolism, and therefore, the kinetics of  $\text{CO}_2$  generation from the individual carbon atom of these compounds might provide evidence for the operation of various pathways in an unknown organism. Preliminary experiments were conducted in which additions of  $1^{14}\text{C}$  lactate,  $2^{14}\text{C}$  lactate,  $1^{14}\text{C}$  acetate and  $2^{14}\text{C}$  acetate were made to Mojave, Phoenix and Meadow soils using

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the single addition mode. Results are shown in Figures 4 - 6.

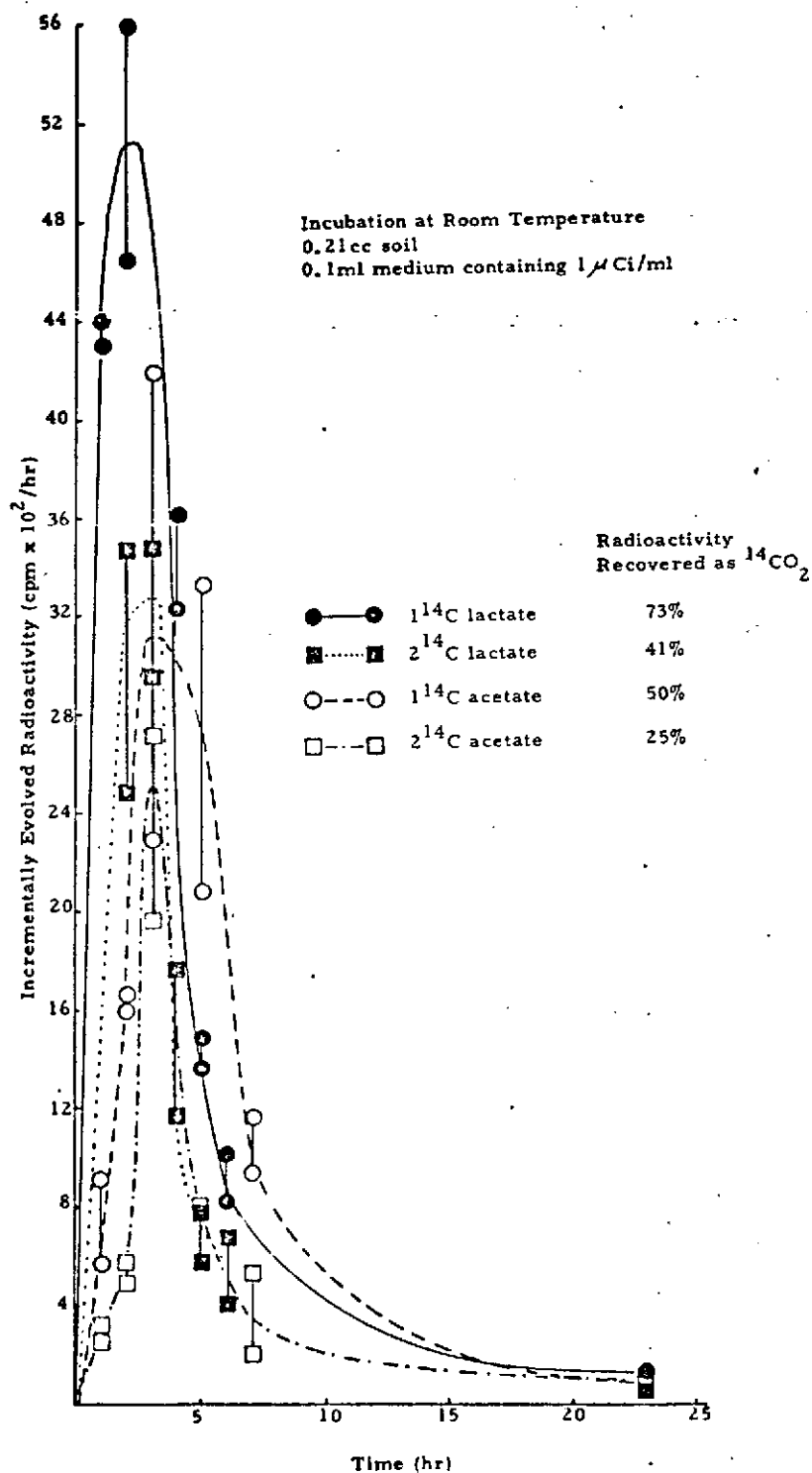
Although the magnitude of response from each substrate was soil dependent, the greatest amount of  $^{14}\text{CO}_2$  evolution for each soil was from  $1^{14}\text{C}$  lactate. The second most active substrate was  $1^{14}\text{C}$  acetate. The results demonstrate the facility with which decarboxylation occurs and that these carbon atoms are primarily expelled as the result of degradation. The fact that acetate produced lesser amounts of  $^{14}\text{CO}_2$  may be due to a greater involvement of this compound in synthetic processes. With the Mojave soil, it is interesting that the  $2^{14}\text{C}$  lactate produced a peak and evolution of  $^{14}\text{CO}_2$ , which was similar to that obtained with  $1^{14}\text{C}$  acetate, possibly indicating that the degradation product of lactate decarboxylation primarily follows a pathway similar to that of acetate. Meadow soil showed similar recoveries of  $^{14}\text{CO}_2$  from  $1^{14}\text{C}$  acetate and  $2^{14}\text{C}$  lactate. However, the kinetics of evolution from these compounds are dissimilar, the  $2^{14}\text{C}$  lactate proceeding at a much slower rate.

The recovery of  $^{14}\text{CO}_2$  from  $^{14}\text{C}$  acetate with Phoenix soil was greater than from  $2^{14}\text{C}$  lactate, possibly indicating that these two substrates are involved in different pathways of metabolism in this soil. The acetate may have become involved in energy yielding pathways, perhaps the citric acid

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Figure 4

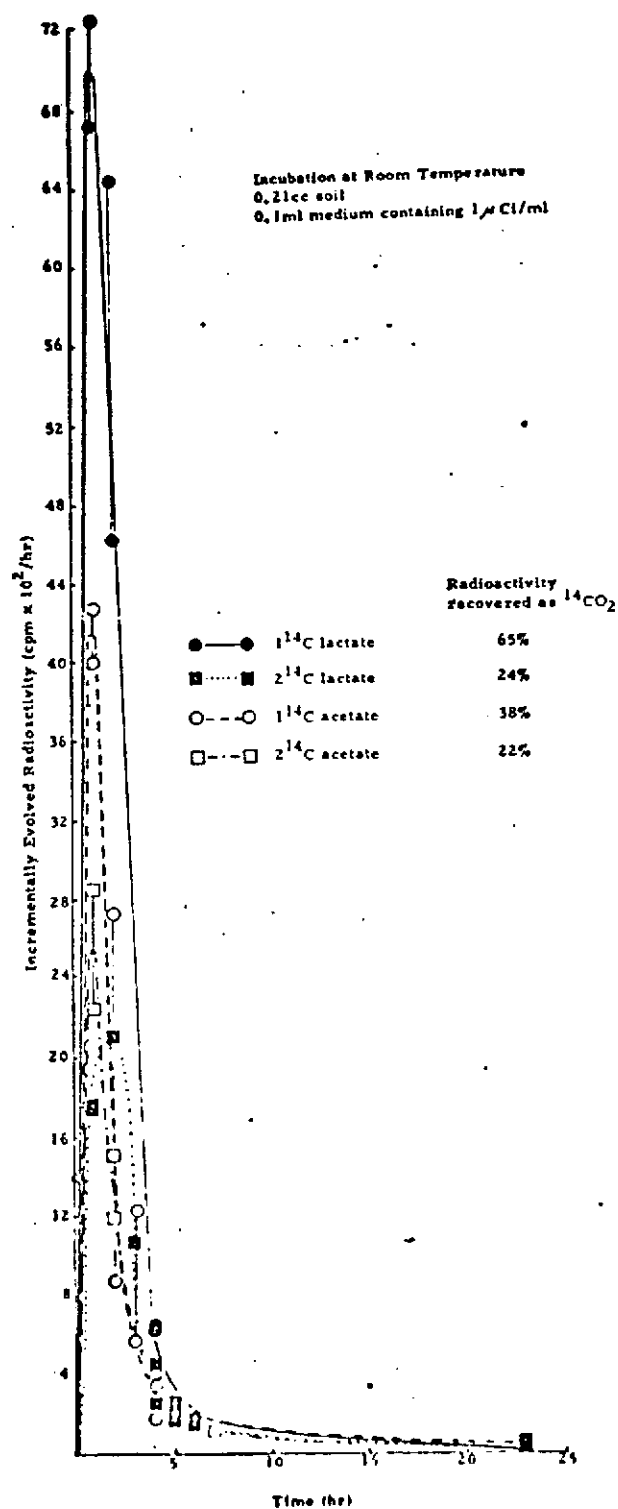
Evolution of  $^{14}\text{CO}_2$  from Specifically Labeled  
 Lactate and Acetate by Mojave Soil



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Figure 5

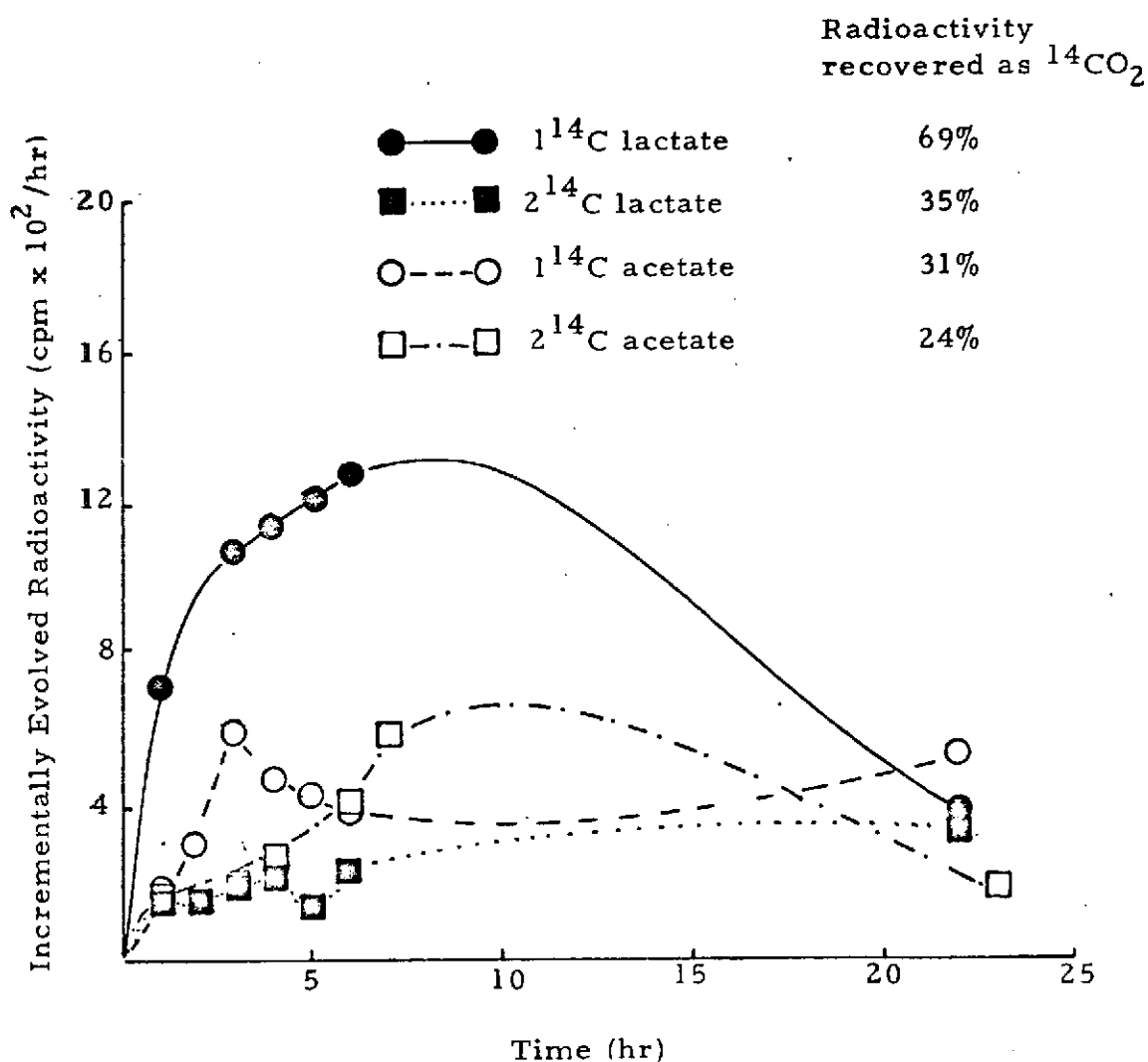
Evolution of  $^{14}\text{CO}_2$  from Specifically Labeled  
 Lactate and Acetate by Phoenix Soil



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Figure 6

Evolution of  $^{14}\text{CO}_2$  from Specifically Labeled  
 Lactate and Acetate by Meadow Soil



Incubation at Room Temperature  
 0.21cc soil  
 0.1ml medium containing  $1\mu\text{Ci/ml}$

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cycle, whereas the two carbon fragment of lactate degradation may have been shunted to synthetic systems.

The establishment of a library of soil profiles will provide a response envelope delineating terrestrial microbial biochemistry. Future responses obtained from Mars can be examined for general conformity with this pattern. Extraordinary differences will serve notice of a novel Martian biochemistry. Correlation with pathways of metabolism of specific substrates by specific organisms or soils with predominant species can provide the first qualitative assessment of the biological organisms found. The control of environmental conditions and substrate selection can initiate comparative biological studies of Martian organisms.

### C. Specificity for D vs. L Configuration

Media to demonstrate specificity for D vs. L were incorporated into the addition series early in the study. A large difference in the  $^{14}\text{CO}_2$  evolution from D vs. L-glucose was obtained. However, requirements for heat sterilization of the media destroyed these substrates making them unsuitable for use in planetary landers. A medium containing UL  $^{14}\text{C}$  D-alanine, 1  $^{14}\text{C}$  D-leucine and 3  $^{14}\text{C}$  D-serine was compared with a medium containing UL  $^{14}\text{C}$  L-alanine, 1  $^{14}\text{C}$  L-leucine and

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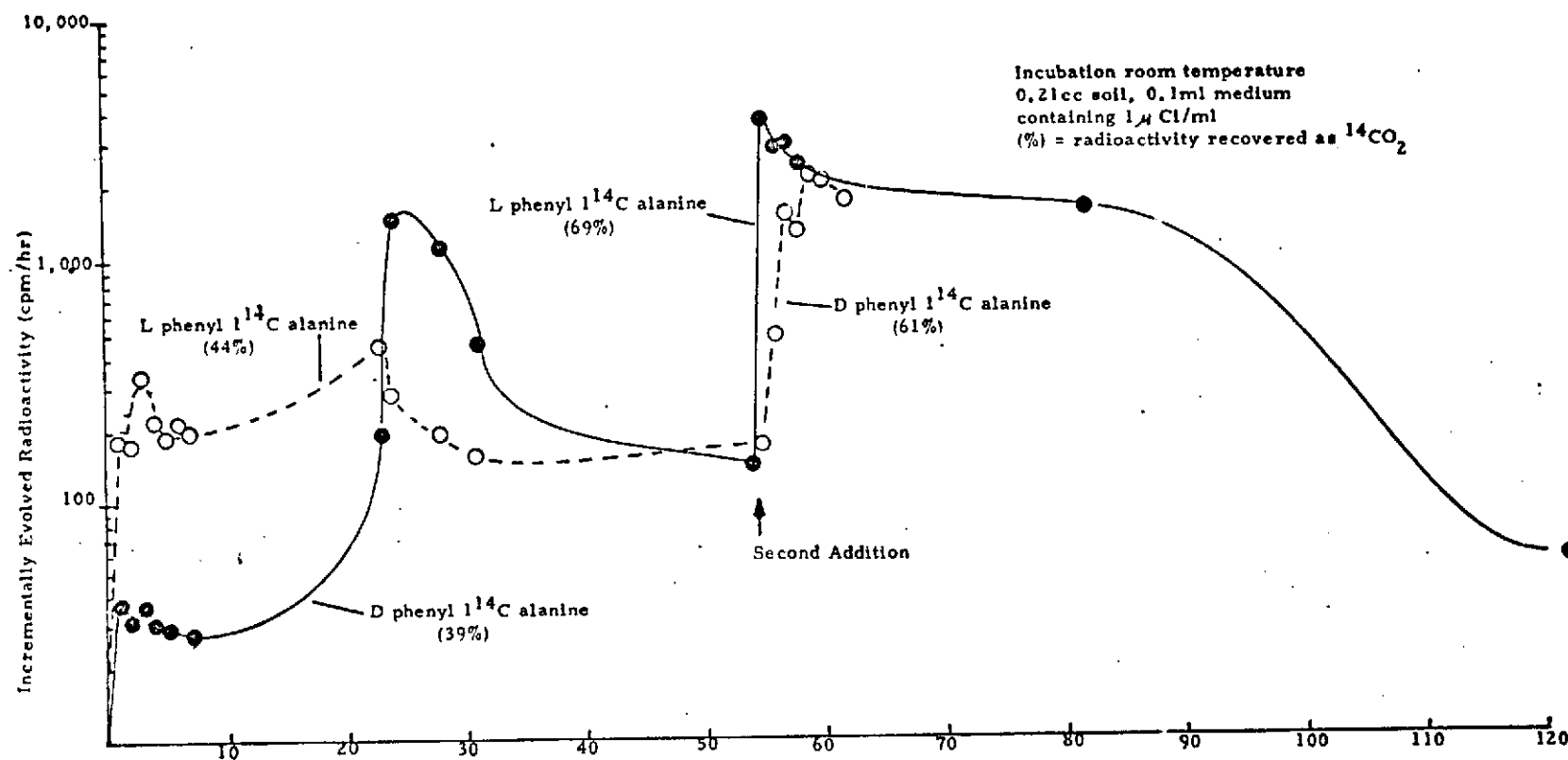
and  $3^{14}\text{C}$  L-serine. Differences in the  $^{14}\text{CO}_2$  evolved from these media were not great. Another possible candidate which was tested is phenylalanine. D-phenyl  $1^{14}\text{C}$  alanine and L-phenyl  $1^{14}\text{C}$  alanine were dosed onto separate samples of Meadow soil. Radioactivity was collected over a period of 54 hours and then a second dose of media was added. Soil initially dosed with D-phenyl  $1^{14}\text{C}$  alanine was dosed the second time with L-phenyl  $1^{14}\text{C}$  alanine, and vice versa. Results, as shown in Figure 7, demonstrate that the L form produced a more rapid response than the D form. It was also observed, however, that the second addition response was greater than the first addition response, independent of the D or L form. The magnitude of response from a first addition of L-phenylalanine was less than a second addition of D-phenylalanine. It was therefore obvious that a first addition of one substrate could not be compared with subsequent additions of another substrate.

An experiment was performed to determine if a prior addition of phenylalanine effects a subsequent response from an addition of phenylalanine. Results shown in Figure 7 indicate a definite influence; however, it was not possible to conclude that phenylalanine specially enhanced the second addition as opposed to the possibility of getting a similar enhancement by the addition



Figure 7

Evolution of  $^{14}\text{CO}_2$  from D and L phenyl  $^{14}\text{C}$  alanine  
by Meadow Soil



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of some other substrate. Would a first addition of a substrate other than phenylalanine also show the same effect? To answer this question, an experiment was conducted in which media containing  $2^{14}\text{C}$  lactate and  $2^{14}\text{C}$  lactate plus unlabeled DL-phenylalanine were dosed onto soil incubated 24 hours and then subsequently dosed with D or L-phenyl  $1^{14}\text{C}$  alanine. Results are shown in Figure 8.

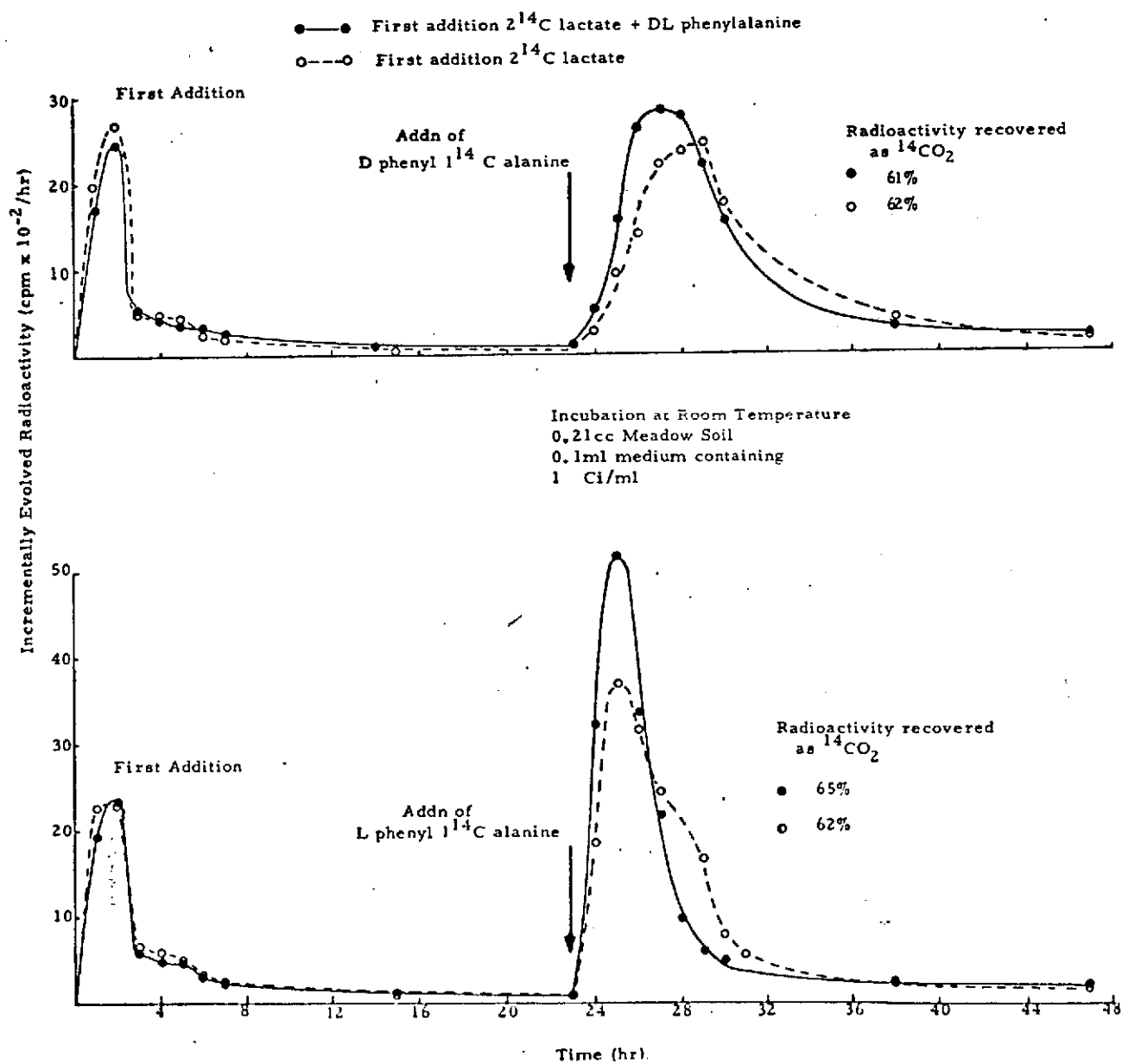
Preincubation of the soil in the presence of DL-phenylalanine enhanced the rate of  $^{14}\text{CO}_2$  evolution from both D and L-phenyl  $1^{14}\text{C}$  alanine added subsequently. However, the L form showed a greater rate and a higher peak than the D form. The yield of  $^{14}\text{CO}_2$  from both the D and L forms was the same with and without a preincubation in DL-phenylalanine. These data show that both forms are utilized, however, the rate of degradation of the D form is much slower and may involve a rate limiting racemase. Enzyme induction would not appear to be involved since preincubation in DL-phenylalanine should have induced the required enzyme system for both forms.

It should also be noted from Figure 8 that the presence of DL-phenylalanine did not effect the evolution of  $^{14}\text{CO}_2$  from  $2^{14}\text{C}$  lactate. These findings suggested that  $^{14}\text{C}$  labeled substrate experiments performed in the multiple addition mode should be

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Figure 8

Evolution of  $^{14}\text{CO}_2$  from D and L phenyl  $^{14}\text{C}$  alanine With  
 and Without Preincubation in phenylalanine



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preceded by a preincubation in that substrate. This could be done by adding unlabeled substrate to the media preceding that  $^{14}\text{C}$  labeled substrate.

### D. Total Colony Counts of Multiple Addition Experiments

It has been shown that a first addition of  $^{14}\text{C}$  labeled medium to soil produces a rate of  $^{14}\text{CO}_2$  evolution which is less than a subsequent addition. This phenomenon is not substrate specific and occurs when the initial substrate differs from the subsequent addition. A possible explanation is that the microorganisms reproduce and, therefore, the second addition of medium is effectively given a larger inoculum.

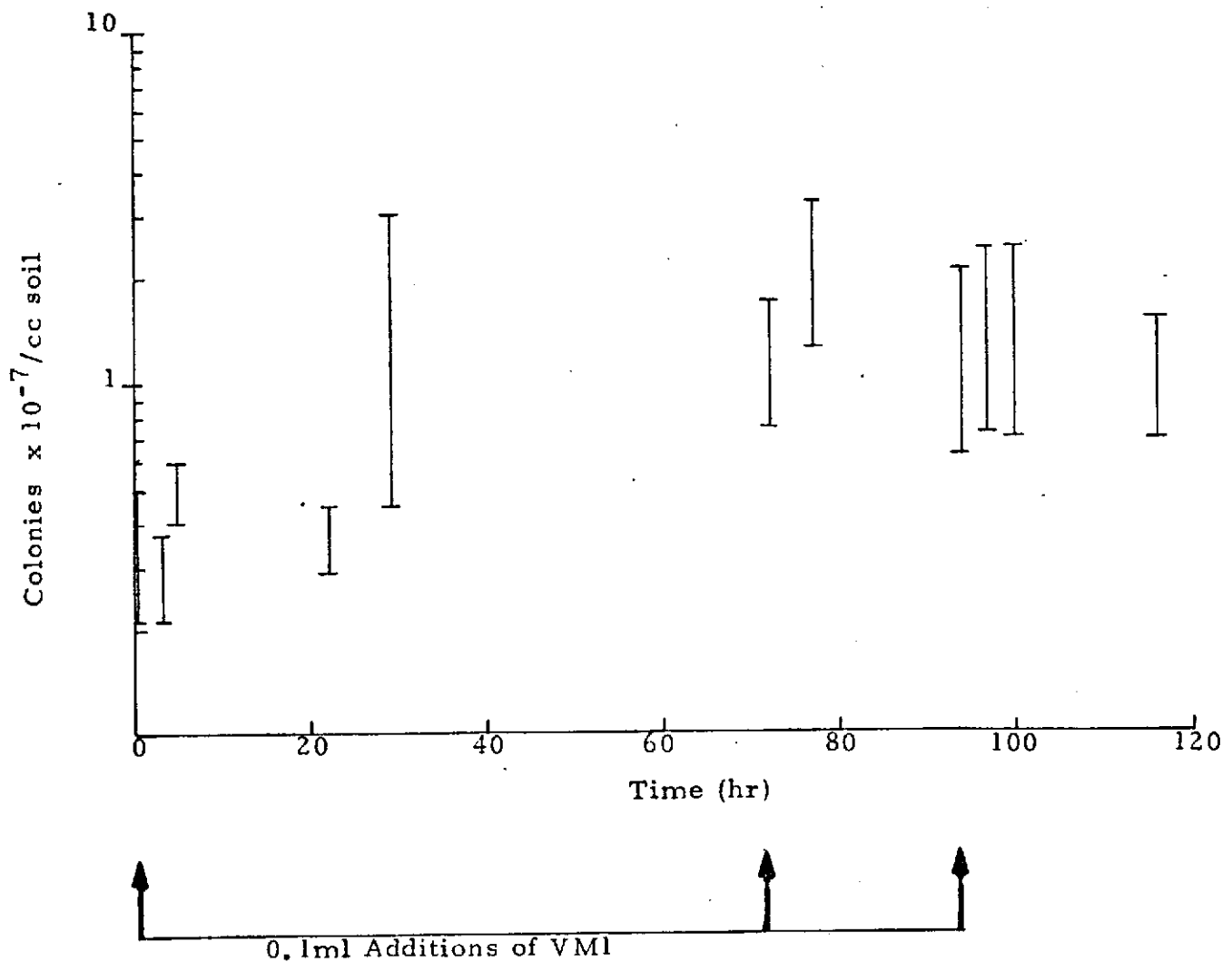
Total plate counts were conducted on a series of soil samples which had been subjected to multiple additions of media. Soil cultures which had received one or more sequential doses of VM1 medium were brought to 2 ml with distilled water, and sonicated to loosen microorganisms from soil particles and break-up clumps. Aliquots of this suspension were diluted and pour plates of nutrient agar prepared. Plates were incubated for six days at room temperature and the total number of colonies counted. Results obtained over a period of 118 hours which included three medium additions, are shown in Figure 9. No

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Figure 9

Number of Colonies Plated from Meadow  
Soil During Multiple Addition of Media

Medium for Plate counts was Nutrient Agar. Plates were incubated at room temperature for 6 days. 0.21cc Meadow Soil was dosed with 0.1ml VM1 and incubated at room temperature. Three soil replicates and four plate count replicates (12 values) were used to establish each bar.



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significant increase in the number of colonies occurred during the first 22 hours; however, a two-five fold increase was found after 24 hours of incubation. The number of colonies found after subsequent medium additions did not appear to change significantly and ranged from  $6 \times 10^7$  to  $3 \times 10^8$  colonies per cc of soil added. Plate counts obtained on a long-term (440 hours) multiple addition experiment which involved the addition of 13 individual substrates (a total of 1.3 ml  $H_2O$ ) showed  $2 \times 10^8$  to  $5 \times 10^8$  colonies/-cc soil. As shown in Figure 9, the addition of a substrate appeared to increase cell numbers slightly during the first few hours of incubation. However, the general population in terms of colony counts remained relatively constant after the second medium addition. It was also noted that the colonies obtained after a long-term multiple addition experiments were morphologically similar to those obtained with fresh soil. Furthermore, the pour plates of these soil suspensions showed that the relative abundance of the various morphologically distinguishable species remained unchanged.

These data suggest that the first substrate addition produces an increase in microorganisms; however, continued incubation and additional substrate additions produce only minor fluctuations in the number of microorganisms. Thus the small increase in

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numbers of organisms may explain in part the greater rate of substrate degradation, by subsequent additions beyond the first. Further, it indicates that the first addition is sufficient to permit growth of the organism to the maximum number. The ecological system will support third and later additions and show no further increase in the rate of  $^{14}\text{CO}_2$  evolution. At present, there is no explanation for the corresponding increase in the yield of  $^{14}\text{CO}_2$  from additions subsequent to the first.

E. Stimulation of  $^{14}\text{CO}_2$  Evolution by Subsequent Substrate Additions

The position of the  $^{14}\text{C}$  carbon atom in the labeled substrate affects not only the rate at which  $^{14}\text{CO}_2$  is produced, but also the total yield of  $^{14}\text{CO}_2$  which occurs. It was suspected that an addition of another substrate may induce the further degradation of a substrate remaining from a previous addition. To test this possibility, a series of  $^{14}\text{C}$  labeled substrates was added to soil, and the evolution of  $^{14}\text{CO}_2$  monitored. Following the peak in  $^{14}\text{CO}_2$  evolution, a second addition of an unlabeled substrate was made. As shown in Figures 10-17, the addition of unlabeled substrate in most cases resulted in a detectible peak in evolution, however, the amount of radioactivity released in this fashion was relatively small. The addition of DL-phenylalanine to

Figure 10

Effect of an Addition of Formate on the Evolution  
of  $^{14}\text{CO}_2$  from  $^{14}\text{C}$  Labeled VMI Medium

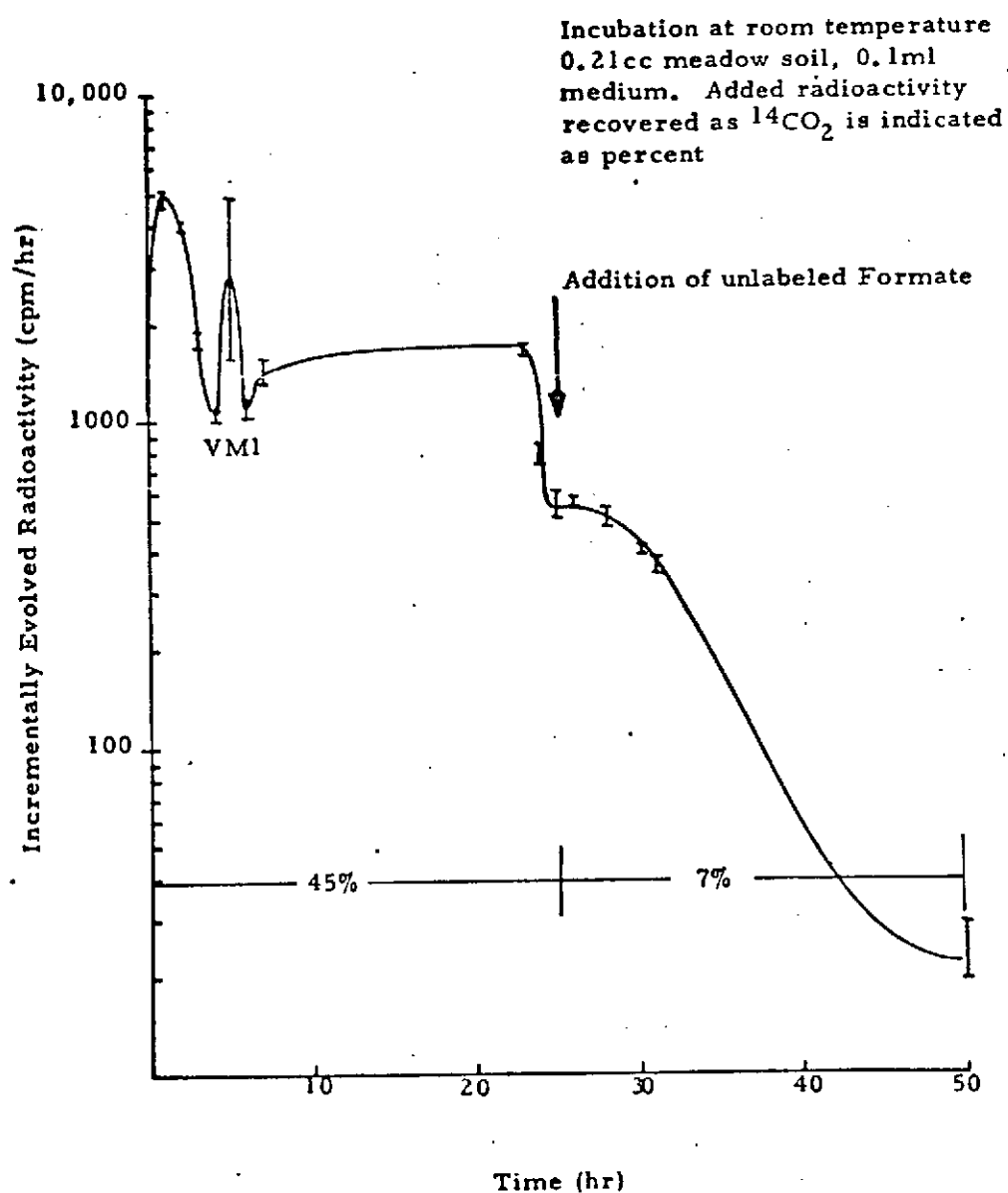




Figure 11

Effect of a Lactate Addition on the  
Evolution of  $^{14}\text{CO}_2$  from  $^{14}\text{C}$  Formate

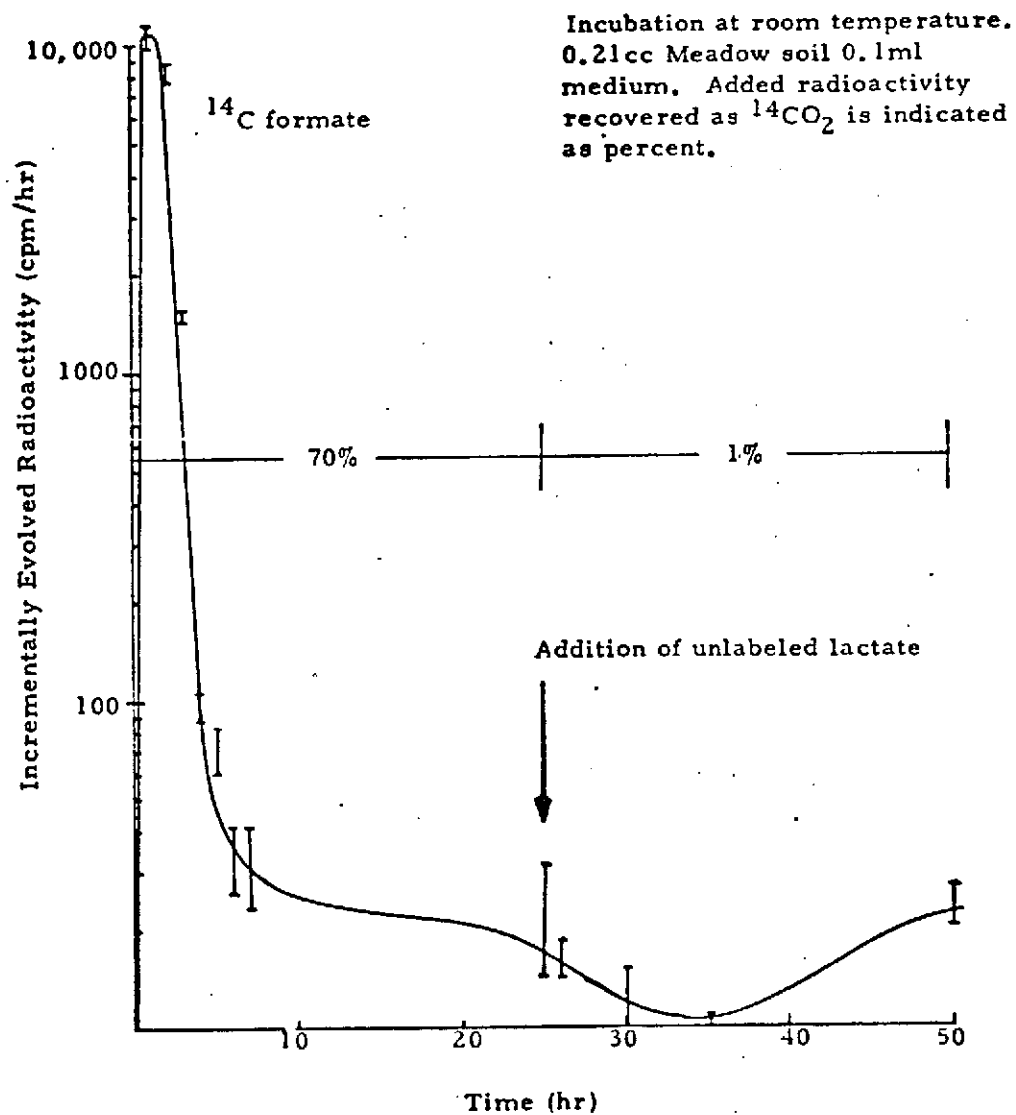
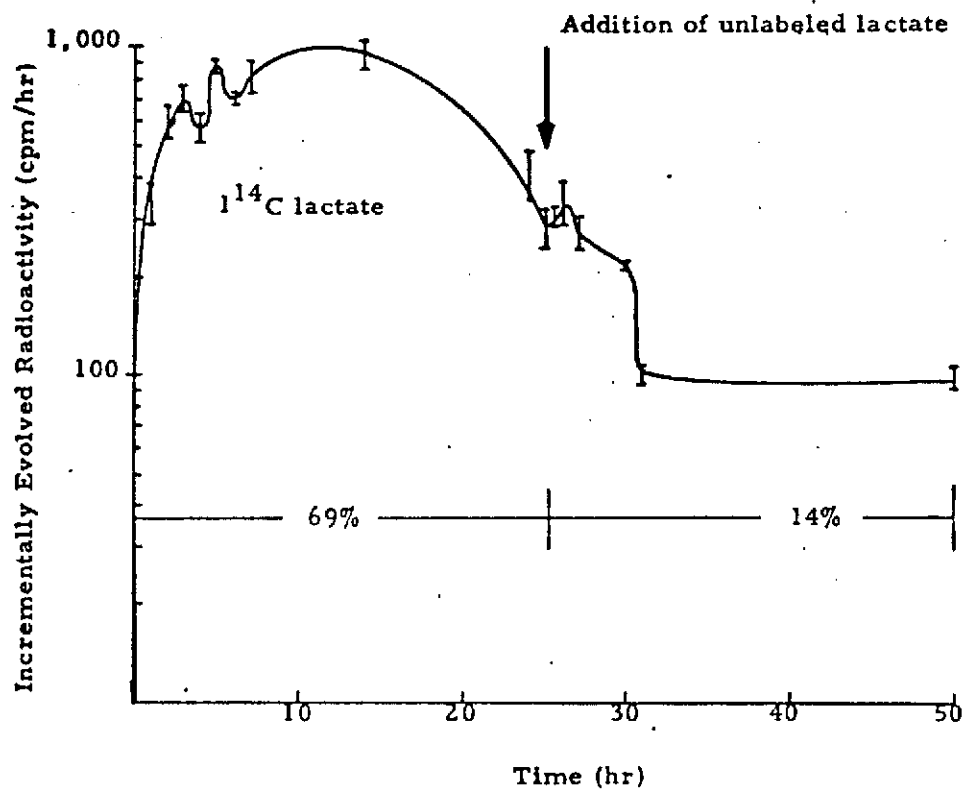


Figure 12

Effect of a Lactate Addition on the Evolution  
of  $^{14}\text{CO}_2$  from  $^{14}\text{C}$  Lactate



Incubation at room temperature  
0.21 cc Meadow soil, 0.1ml  
medium. Added radioactivity  
recovered as  $^{14}\text{CO}_2$  is indicated  
as percent.

Figure 13

Effect of a Second DL-Lactate Addition on the  
 Evolution of  $^{14}\text{CO}_2$  from 2,3- $^{14}\text{C}$  DL-Lactate

Incubation at room temperature  
 0.21cc Meadow Soil, 0.1ml  
 medium. Added Radioactivity  
 recovered as  $^{14}\text{CO}_2$  is indicated  
 as percent.

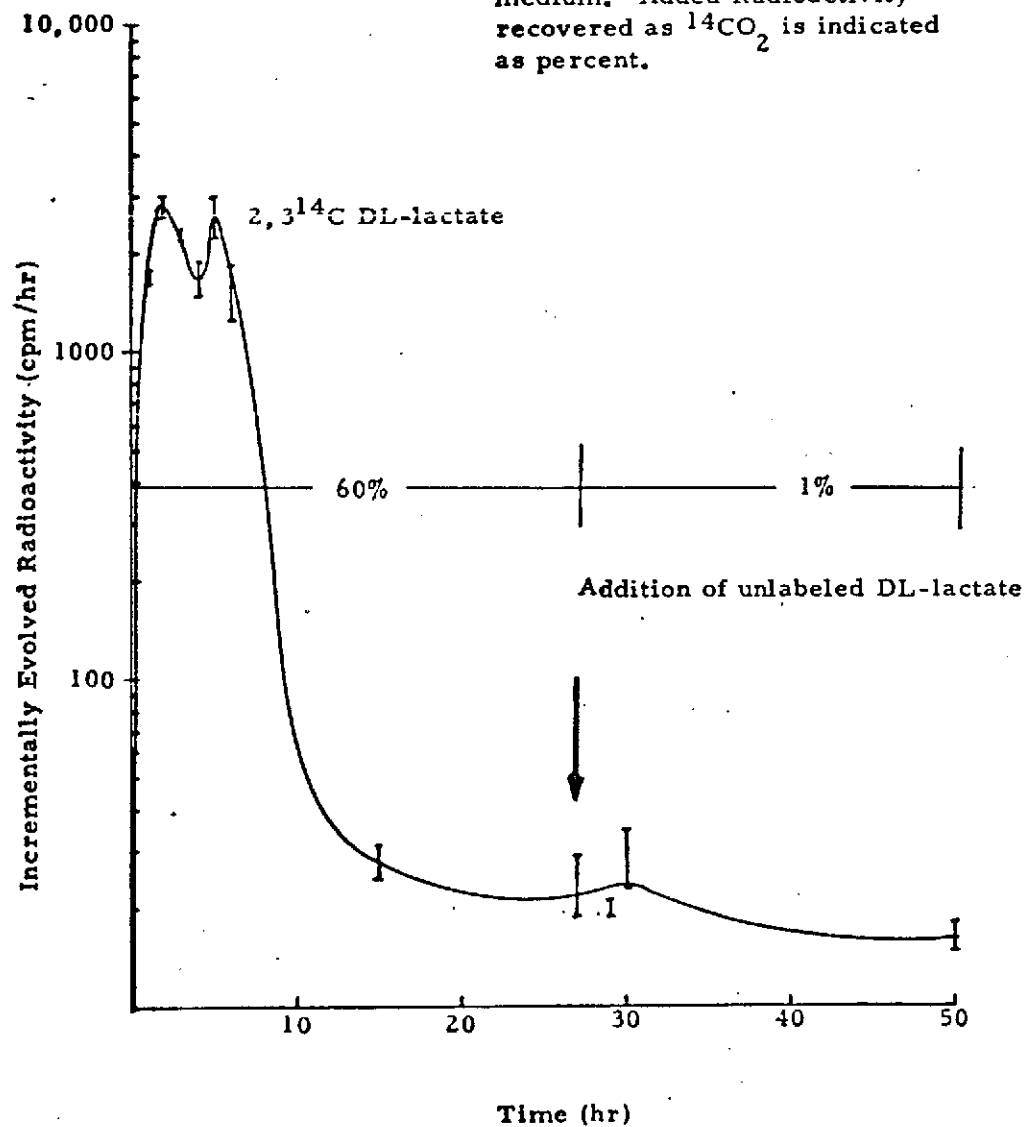


Figure 14

Effect of an Acetate Addition on the Evolution  
of  $^{14}\text{CO}_2$  from  $2^{14}\text{C}$  Lactate

Incubation at room temperature  
0.21cc Meadow Soil, 0.1 ml  
medium. Added radioactivity  
recovered as  $^{14}\text{CO}_2$  is indicated  
as percent.

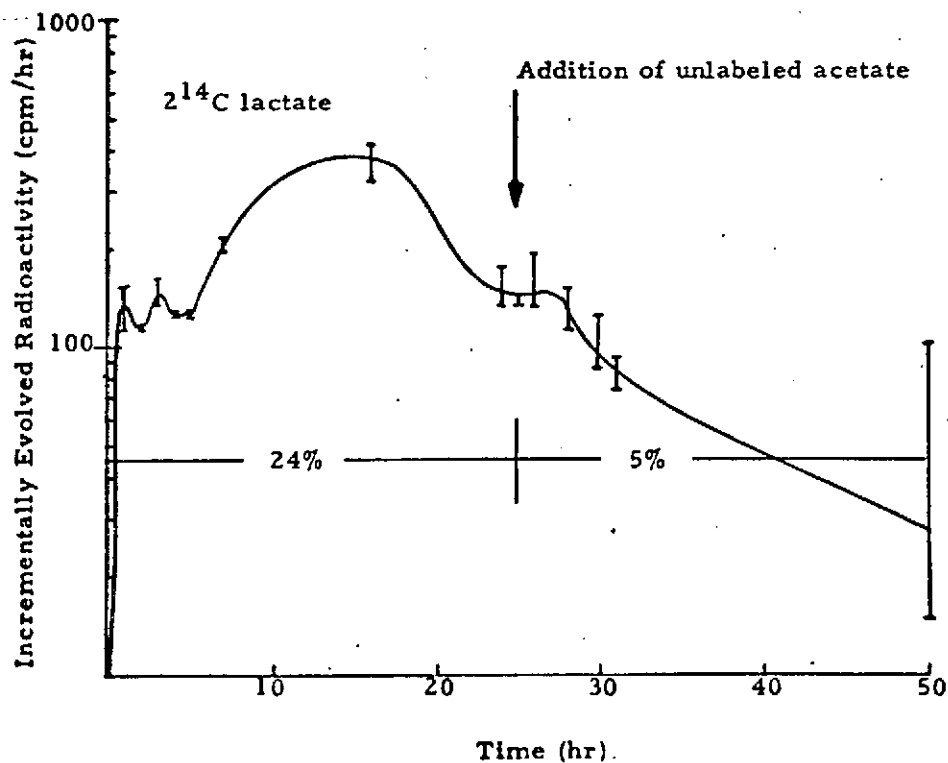


Figure 15

Effect of Second Addition of Acetate on the  
Evolution of  $^{14}\text{CO}_2$  from  $^{14}\text{C}$  Acetate

Incubation at room temperature  
0.21 cc Meadow Soil, 0.1 ml  
medium. Radioactivity recovered  
as  $^{14}\text{CO}_2$  is indicated as percent.

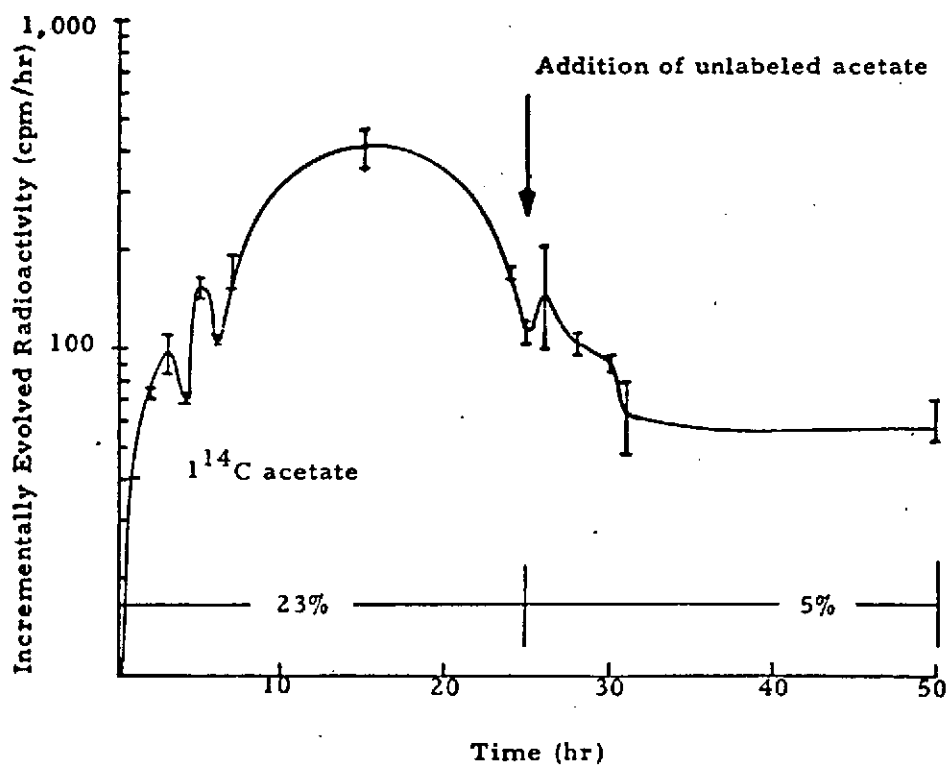


Figure 16

Effect of an Addition of DL Phenylalanine on the  
Evolution of  $^{14}\text{CO}_2$  from  $2^{14}\text{C}$  Acetate

Incubation at room temperature  
0.21 cc Meadow Soil, 0.1ml  
medium. Radioactivity recovered  
as  $^{14}\text{CO}_2$  is indicated as percent.

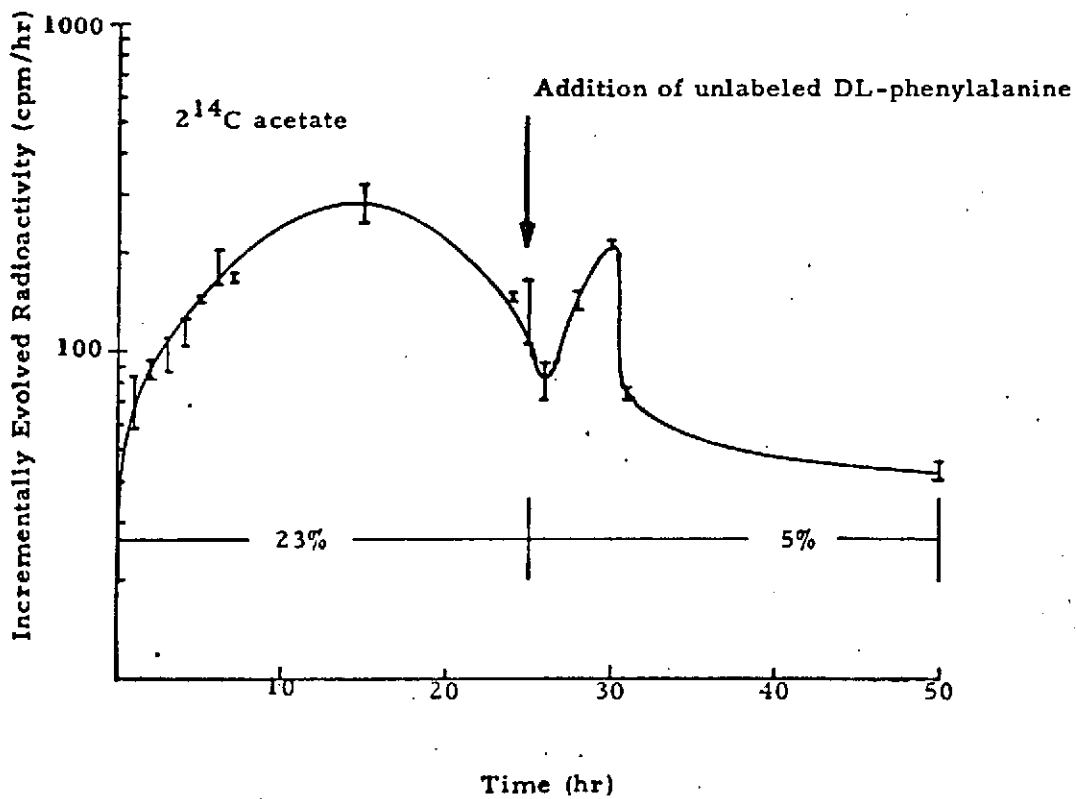
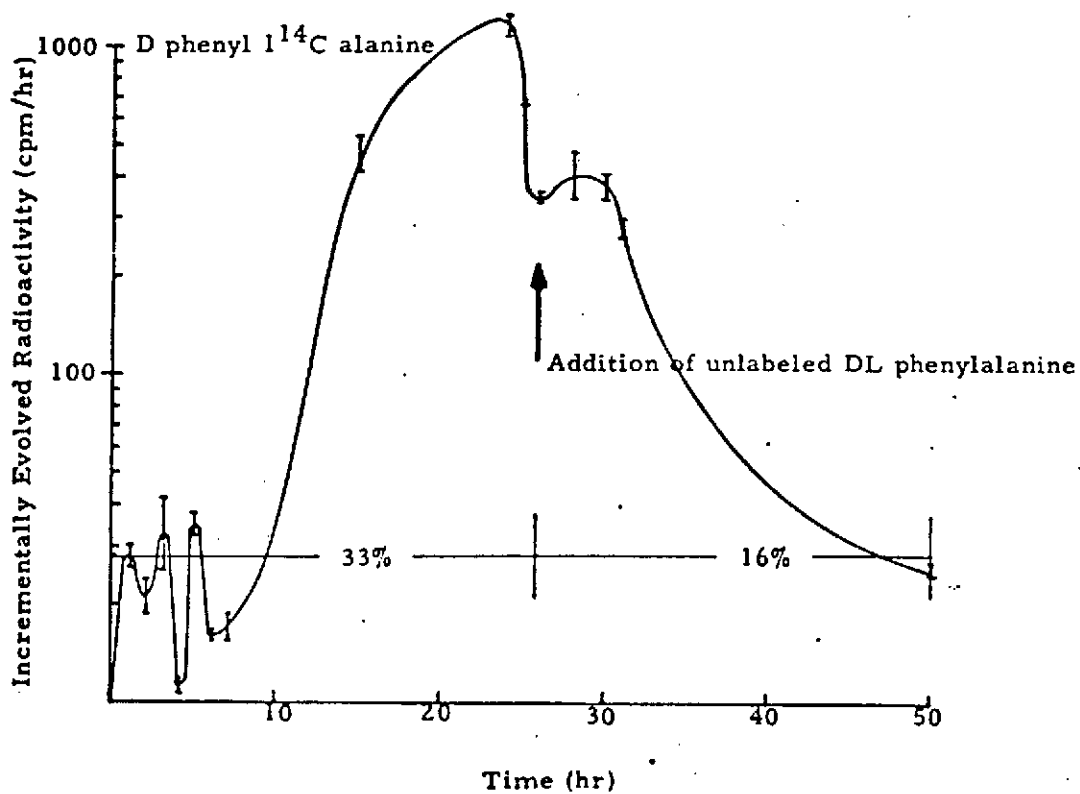


Figure 17

Effect of an Addition of DL Phenylalanine on the  
Evolution of  $^{14}\text{CO}_2$  from D phenyl  $1^{14}\text{C}$  Alanine

Incubation at room temperature  
0.21cc Meadow Soil, 0.1ml  
medium. Radioactivity recovered  
as  $^{14}\text{CO}_2$  is indicated as percent.



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soils previously dosed with  $2^{14}\text{C}$  lactate or D phenyl  $1^{14}\text{C}$  alanine produced the greatest secondary peak in evolution of radioactivity. However, it should be noted that the evolution of  $^{14}\text{CO}_2$  was by soils which were not medium enhanced, i. e., the  $^{14}\text{C}$  labeled substrate was the first addition. Thus, following the plateau of a first addition, additional radioactivity from that substrate may be evolved by the addition of other substrates. The stimulation of  $^{14}\text{CO}_2$  evolution from a  $^{14}\text{C}$  labeled substrate by a subsequent substrate addition is less likely under conditions of the multiple addition. This may correlate with the fact that the recovery of  $^{14}\text{CO}_2$  from added labeled substrates is higher than from corresponding first additions.

Data (Figures 10-17) shows that in the multiple addition mode,  $^{14}\text{CO}_2$  evolution which occurs after an addition of  $^{14}\text{C}$  labeled substrate arises primarily from that substrate and not from  $^{14}\text{C}$  labeled substrates added earlier.

### F. Multiple Addition Substrate Series

A multiple addition series which included  $^{14}\text{C}$  labeled VMI,  $1^{14}\text{C}$  formate,  $1^{14}\text{C}$  acetate,  $2^{14}\text{C}$  acetate,  $1^{14}\text{C}$  lactate,  $2\text{-}3^{14}\text{C}$  lactate,  $2^{14}\text{C}$  lactate, L phenyl  $1^{14}\text{C}$  alanine, D phenyl  $1^{14}\text{C}$  alanine,  $1^{14}\text{C}$  glutamate,  $2^{14}\text{C}$  glutamate,  $3\text{-}4^{14}\text{C}$  glutamate and  $5^{14}\text{C}$  glutamate was conducted. Media containing these



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specifically labeled substrates were sequentially added to a single soil sample. Two independent series of media were investigated, both of which included the same  $^{14}\text{C}$  labeled substrates. Series (A) was composed of media which contained one  $^{14}\text{C}$  labeled substrate and no unlabeled substrates. Series (B) was composed of media which contained one  $^{14}\text{C}$  labeled substrate and, in some cases, an unlabeled substrate. The media used in the two test series are listed in Table 1 in the order in which they were added to the soil. As shown in Series (B), a change in substrate was preceded by a  $^{14}\text{C}$  labeled medium which also contained the unlabeled substrate scheduled to follow as a  $^{14}\text{C}$  labeled substrate.

By using the medium Series (B), a soil culture is not dosed with a  $^{14}\text{C}$  labeled substrate with which it is unfamiliar. The induction of enzymes no longer is involved in the response from a given substrate, and differences in the rate and yield of  $^{14}\text{CO}_2$  may be interpreted more easily in terms of metabolic pathways.

Results of multiple addition tests on Meadow soil utilizing the media additions listed in Series (A) and Series (B) are shown in the two foldout graphs (pages 32, 33). Recovery of

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Table 1

Media \* Used for Multiple Addition Tests  
 Listed in Order of Addition

<u>Code</u>	<u>Series (A)</u>	<u>Series (B)</u>
VMI	VMI **	VMI *
F	1 <sup>14</sup> C formate	1 <sup>14</sup> C formate + acetate
A <sub>1</sub>	1 <sup>14</sup> C acetate	1 <sup>14</sup> C lactate
A <sub>2</sub>	2 <sup>14</sup> C acetate	2 <sup>14</sup> C acetate + DL-lactate
L <sub>1</sub>	1 <sup>14</sup> C L-lactate	1 <sup>14</sup> C L-lactate
L <sub>3</sub>	2-3 <sup>14</sup> C L-lactate ***	2-3 <sup>14</sup> C L-lactate
L <sub>2</sub>	2 <sup>14</sup> C L-lactate	2 <sup>14</sup> C L-lactate + DL phenylalanine
PA <sub>L</sub>	L-phenyl 1 <sup>14</sup> C alanine	L phenyl 1 <sup>14</sup> C alanine
PA <sub>D</sub>	D-phenyl 1 <sup>14</sup> C alanine	D-phenyl 1 <sup>14</sup> C alanine
G <sub>1</sub>	1 <sup>14</sup> C DL-glutamate	1 <sup>14</sup> C DL-glutamate
G <sub>2</sub>	2 <sup>14</sup> C DL-glutamate	2 <sup>14</sup> C DL-glutamate
G <sub>3,4</sub>	3-4 <sup>14</sup> C DL-glutamate	3-4 <sup>14</sup> C DL-glutamate
G <sub>5</sub>	5 <sup>14</sup> C DL-glutamate	5 <sup>14</sup> C DL-glutamate

\* All substrates at a concentration of  $2.5 \times 10^{-4}$ M.

\*\* VMI, 1  $\mu$ Ci/ml composed of UL<sup>14</sup>C L-alanine, UL<sup>14</sup>C D-alanine, UL<sup>14</sup>C glycine, <sup>14</sup>C formate, UL<sup>14</sup>C DL-lactate, UL<sup>14</sup>C glycolic acid. (Total radioactivity 3.4  $\mu$ Ci/ml).

\*\*\* Data are obtained for 3<sup>14</sup>C lactate by multiplying cpm for 2-3<sup>14</sup>C lactate (1  $\mu$ Ci/ml) x 2 and subtracting cpm for 2<sup>14</sup>C lactate (1  $\mu$ Ci/ml).

3<sup>14</sup>C lactate is not commercially available except as a special item.

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$^{14}\text{CO}_2$  is listed in Table 2. Four replicates were determined for each series.

This experiment, which was monitored continuously for 440 hours and involved 13 medium additions, demonstrates the reproducibility of the multiple addition system. Series (B) compared almost identically to Series (A) thereby showing that the presence of unlabeled substrates did not affect the kinetics or total cumulative amount of  $^{14}\text{CO}_2$  evolved from the substrate with which they were mixed. The fact that little or no effect was induced by the unlabeled substrates is not considered to be justification for excluding them. The possibility of either enhancement or inhibition exists, however, the probability of the former would seem to be greater. Dilution of the radioactivity by the unlabeled substrate could foreseeably decrease the rate of  $^{14}\text{CO}_2$  evolution from the labeled substrate, however, such an effect was not observed with Meadow soil. Continued soil testing should provide further justification for inclusion or exclusion of the unlabeled substrates.

### Discussion of Meadow Soil Results

In the experimental test shown for Meadow soil, the results obtained from the test series of specifically labeled substrates provides the following rationale for speculation concerning the predominating biochemical pathways operating.

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Table 2

Recovery\* of Added Radioactivity

Substrate Code	Recovery (%)	
	Series (A)	Series (B)
VMI	29	32
F	94	90
A <sub>1</sub>	68	74
A <sub>2</sub>	39	35
L <sub>1</sub>	101	100
L <sub>3</sub>	9	5
L <sub>2</sub>	21	23
PA <sub>L</sub>	62	63
PA <sub>D</sub>	76	85
G <sub>1</sub>	81	79
G <sub>2</sub>	54	51
G <sub>3-4</sub>	16	18
G <sub>5</sub>	58	57

\* Includes all radioactivity trapped with Ba(OH)<sub>2</sub> until the next <sup>14</sup>C labeled substrate addition.

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VMI medium was merely used to enhance the soil.

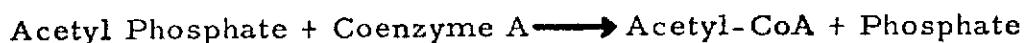
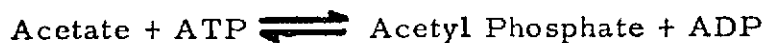
$^{14}\text{C}$  formate,  $1^{14}\text{C}$  acetate and  $1^{14}\text{C}$  lactate all produced a rapid high yield of  $^{14}\text{CO}_2$ . From results shown in Figure 10 (stimulation of  $^{14}\text{CO}_2$  evolution from VMI by addition of formate), it can be assumed that five to ten percent of the total collected radioactivity in Series (A) and (B) for formate was due to VMI. Therefore, the actual recovery of formate is probably approximately 85 percent. This means that the greatest  $^{14}\text{CO}_2$  yield occurred with lactate most likely via oxidation to pyruvate followed by decarboxylation of pyruvate to yield  $\text{CO}_2$  and acetyl-CoA. Several pathways for utilization or further degradation of the acetyl-CoA might be considered. Foremost is the citric acid cycle. Condensation of acetyl-CoA with oxaloacetate would cause the carbon atoms of the acetyl-CoA to be liberated beginning with the second turn of the cycle. Both carbon atoms would rotate to positions for decarboxylation at the same time due to passage through succinate, a symmetrical compound (1). Therefore, if degradation of acetyl-CoA proceeded primarily via the citric acid cycle, equal yields of  $^{14}\text{CO}_2$  from 2 and 3  $^{14}\text{C}$  lactate would be expected. Data represented graphically and recoveries given in Table 2 do not support this route of metabolism. From two-four fold more  $^{14}\text{CO}_2$  was recovered from the 2  $^{14}\text{C}$  lactate than from the 3  $^{14}\text{C}$  lactate. Following

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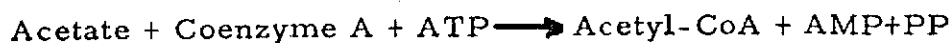
the rapid and complete degradation of  $1^{14}\text{C}$  lactate to  $^{14}\text{CO}_2$ , a small fraction of the remaining two carbon fragments, most likely acetyl-CoA, may enter the citric acid cycle, but the bulk of this compound must be involved in synthetic pathways (amino acid or lipid metabolism). The accumulation of a two or one carbon byproduct is unlikely in the presence of the highly versatile soil population, and data obtained on formate and acetate degradation also argue against this possibility. Results obtained with  $1^{14}\text{C}$  acetate and  $2^{14}\text{C}$  acetate support the explanation that the citric acid cycle is not the exclusive pathway for the utilization of acetyl-CoA.

The degradation of acetate requires activation to acetyl-CoA. This activation requires energy and may proceed via the following:

### Acetokinase



### Aceto-CoA-Kinase



Since the degradation of lactate and acetate probably involves a common intermediate, acetyl-CoA, the degradation rate and yield of



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$^{14}\text{CO}_2$  from  $2^{14}\text{C}$  lactate and  $3^{14}\text{C}$  lactate might be expected to compare with  $1^{14}\text{C}$  acetate and  $2^{14}\text{C}$  acetate, respectively. However, the yield of  $\text{CO}_2$  from acetate was much greater than from the two and three carbon atoms of lactate. This would imply that a greater percentage of acetyl-CoA from acetate had been used for energy yielding metabolism, perhaps via the citric acid cycle, than from the acetyl-CoA derived from lactate.

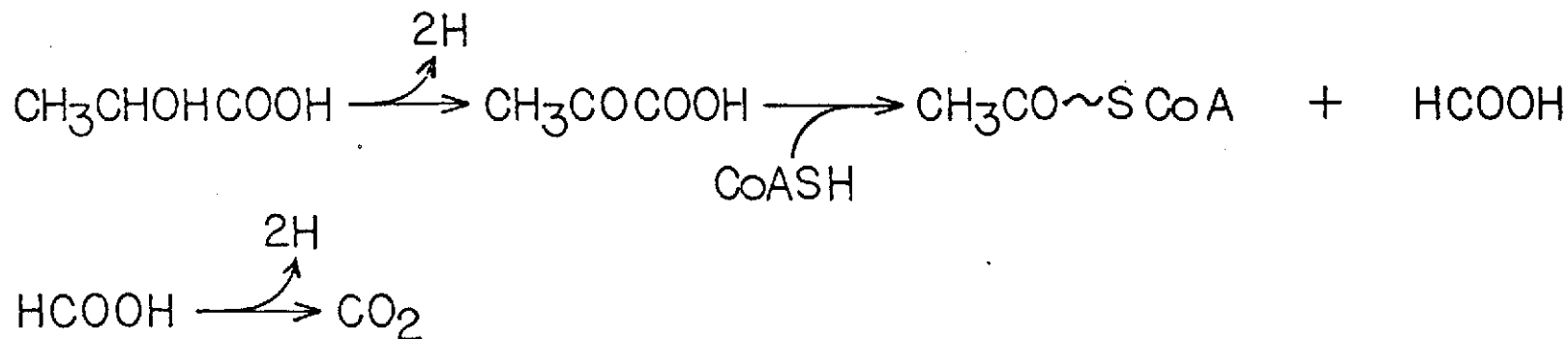
Energy considerations of lactate vs. acetate metabolism may provide an explanation. The most likely schemes for production of acetyl-CoA from the two compounds is as shown in Figure 18. Lactate dehydrogenation results in the production of reduced cofactor. A clastic split of pyruvate to acetyl-CoA would produce formate, an additional source of reduced cofactor. In an aerobic system, these two reduced cofactors might produce a net gain of six ATP moles per mole of acetyl-CoA produced. Activation of acetate on the other hand, leads to a net loss of one mole of ATP per mole of acetyl-CoA produced.

It might easily be reasoned therefore, that the same population of organisms would process acetyl-CoA from lactate differently than acetyl-CoA from acetate. In the former, a net

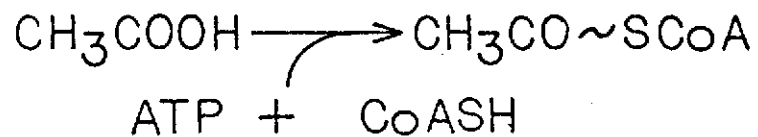
Figure 18

Schemes for Production of Acetyl-CoA  
from Lactate and Acetate

LACTATE



ACETATE



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gain in six ATP would allow for a high percentage of the acetyl-CoA to be directed toward cellular carbon needs, i. e., protein and lipid synthesis. The energy requiring activation of acetate would cause a greater percentage of that acetyl-CoA to be directed through energy producing pathways which ultimately lead to the generation of  $\text{CO}_2$ .

The pattern of specifically labeled glutamate provides evidence for the operation of the citric acid cycle. Therefore, energy production from acetyl-CoA could be produced by that pathway.

The D and L phenyl  $^{14}\text{C}$  alanine provided evidence of specificity for the L form over the D form, but the difference in the rate of utilization of these two forms was not great.

### G. Design and Rationale for the Advanced Labeled Release

A tentative design and rationale for the advanced labeled release experiment has been produced. The concept involves the sequential addition of a series of  $^{14}\text{C}$  labeled substrates to test chambers which are initially charged with a single addition of test soil. The sequential additions of substrates are accompanied by specified environmental conditions such as temperature and gas above the soil culture. Each chamber constitutes an

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independent aspect of the overall test series, which is performed in a designated sequence.

Chamber 1 - containing viable soil allows for performance of the labeled release experiment under various atmospheric conditions. Results of this series should provide information on which atmosphere gives the optimum response and which might serve to control growth) and therefore dictates the atmospheric conditions which should be used for the other three test chambers. The  $^{14}\text{C}$  labeled medium to be used in this series is VMI, which has undergone rigorous testing as a part of the current Viking development. The gaseous atmospheres, which might be studied in this series are  $\text{CO}_2$ ,  $\text{CO}$ ,  $\text{H}_2$ ,  $\text{N}_2$  and 20 percent  $\text{O}_2$  (air). Current experimental laboratory methods for conducting the labeled release involves  $\text{CO}_2$  collection with  $\text{Ba}(\text{OH})_2$ , therefore testing under an atmosphere of  $\text{CO}_2$  and  $\text{CO}$  are not feasible. An alternative detection system (ionization chamber or solid state) would be required.

Demonstration of the methodology has been performed using atmospheres of air and  $\text{N}_2:\text{H}_2$  (90 percent: 10 percent). The rationale for determining the sequence of gases will be to

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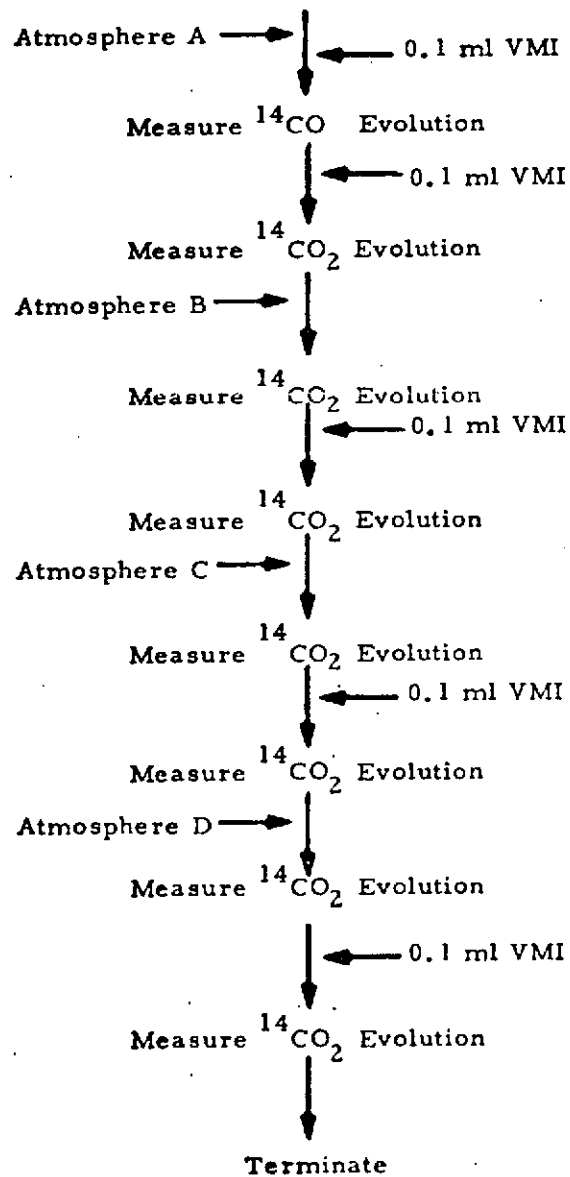
proceed from the theoretically most optimal to the least optimal. Therefore, the latter conditions constitute a measure of inhibition. A schedule of medium additions and atmospheres to be used for demonstration purposes with terrestrial soil is given in the addition sequence for Chamber 1 (Figure 19). An initial temperature of 20°C has been selected for demonstration testing of the atmospheres. An arbitrary temperature selection would also be required for the initial Mars test. Some information should be provided by the Viking results, however, if not, a temperature well within the ambient range would be designated for the Chamber 1 tests.

As shown in the addition sequence for Chamber 1, two additions of VMI medium are added at the initial temperature. Experiments have shown that a first addition of medium to soil shows different kinetics than a second or subsequent addition. Presumably, this difference is due to an increase in the number of organisms and/or conditioning of the organisms and it is important for comparative purposes that each of the atmospheres are tested on preconditioned organisms. Therefore, a double addition must be made initially. Aside from this requirement, however, this first and second addition constitutes a specific

Figure 19

Chamber I  
Addition Sequence  
Atmosphere Test

Ambient Temperature, Viable Soil (0.21cc)



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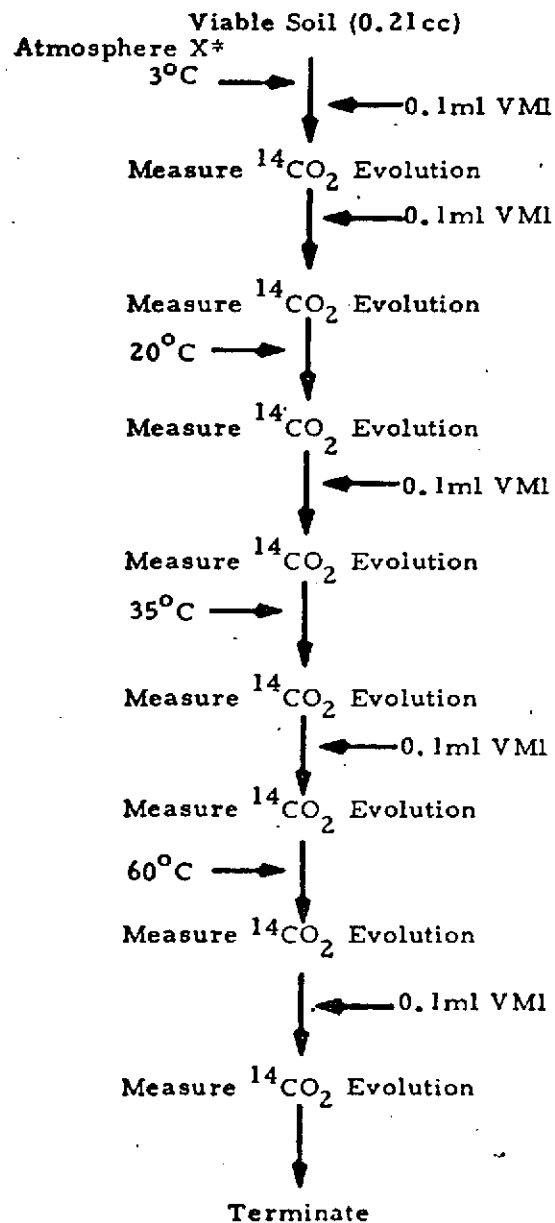
test for medium induced biological activity. The difference in kinetics between a first and second addition is strictly a biological phenomenon, and therefore, provides valuable information.

That a first medium addition increased cell numbers or induced degradation systems would be a highly interesting finding.

Chamber 2 - containing viable soil is used to establish the temperature optimum and range of activity of organisms in a test soil. As shown in Figure 20, the addition sequence for Chamber 2, VMI medium is added sequentially to test soil and each addition is preceded by an increase in the incubation temperature. Temperatures to be used for Martian conditions may or may not coincide with the demonstration procedure temperature of  $3^{\circ}\text{C}$ ,  $20^{\circ}\text{C}$ ,  $35^{\circ}\text{C}$  and  $60^{\circ}\text{C}$ , which have been studied. The  $3^{\circ}\text{C}$  temperature is within the range of Martian ambient temperature, yet above freezing for the medium. Terrestrial soil produces a signal at this temperature and some psychrophiles might show an optimum. The  $20^{\circ}\text{C}$  temperature is approximately the upper limit for the Martian ambient temperature (although  $30^{\circ}\text{C}$  may be experienced in the hot model of the planet). Many soil organisms are active at this temperature. The  $35^{\circ}\text{C}$  temperature is optimum for many terrestrial organisms. Most organisms which inhabit warmblooded animals have their optimum

Figure 20

Chamber 2  
 Addition Sequence  
 Temperature Test



\*Atmosphere selected on basis of Chamber 1 results



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at this temperature and many of these organisms are found in soil.

Thermophiles are active at 60<sup>o</sup> C. Soils thusfar tested at 60<sup>o</sup> C showed decreased activity. Such a temperature would be expected to inhibit Martian organisms and therefore the test, if used on Mars, should provide evidence for inhibition or cidal effects of heat.

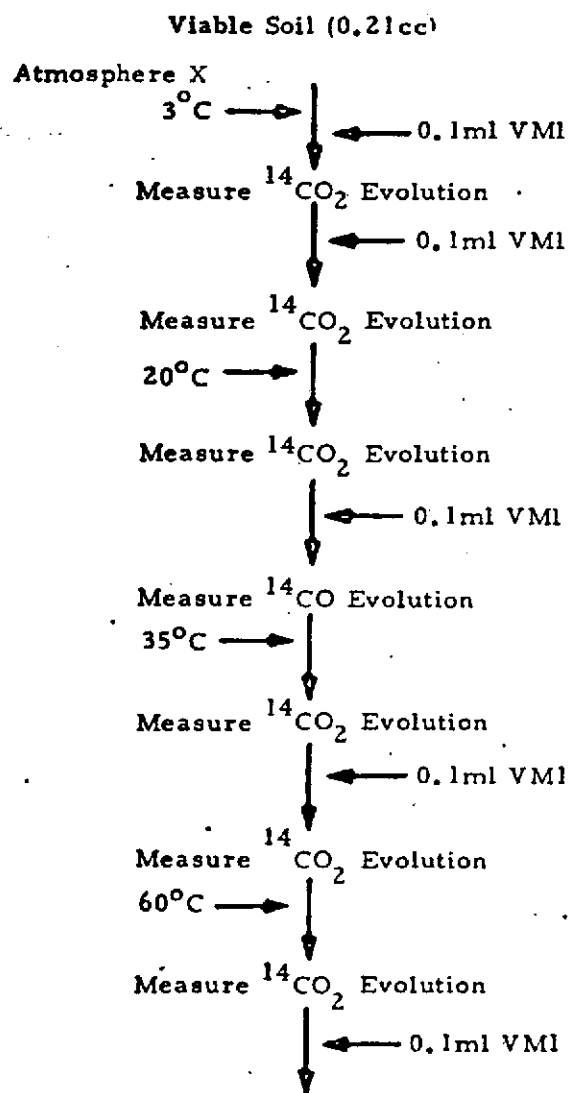
As with Chamber 1, two successive media additions are made at the first temperature. As described for Chamber 1, these additions are necessary and constitute a repeat of results obtained for Chamber 1 (additions 1 and 2) at a different temperature.

The temperature optimum (that which produced the highest rate and cumulative evolution) will be selected for further tests with Chambers 3 and 4.

Chamber 3 - (Figure 21) will be charged with soil which has been presterilized. A series of medium additions and incubation conditions will be imposed. The results of Chamber 3 will serve as a nonbiological control for interpretation of results from Chambers 1, 2 and 4. The sterile medium series is tested prior to the viable medium series in order to provide baseline

Figure 21

Chamber 3  
Addition Sequence  
Sterile Control

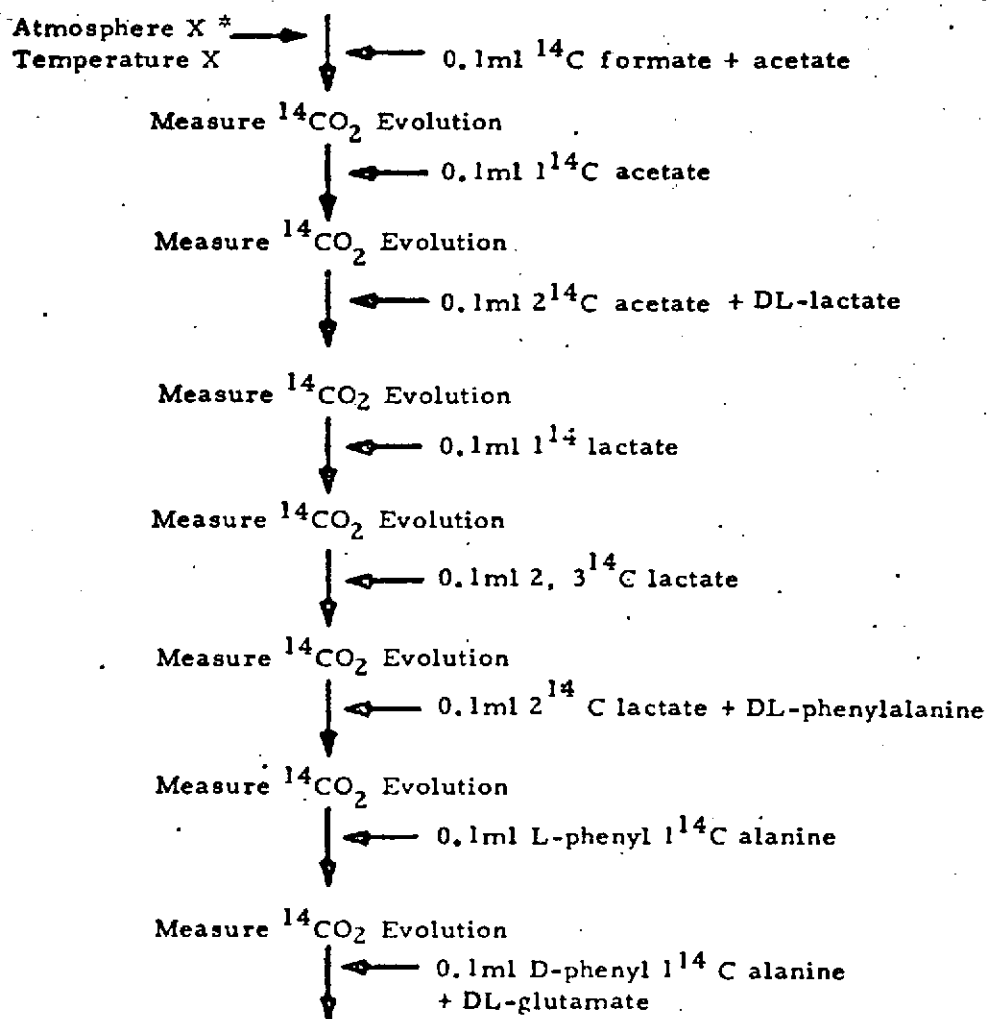


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Figure 21  
 (continued)

Chamber 3  
 Addition Sequence  
 Sterile Control

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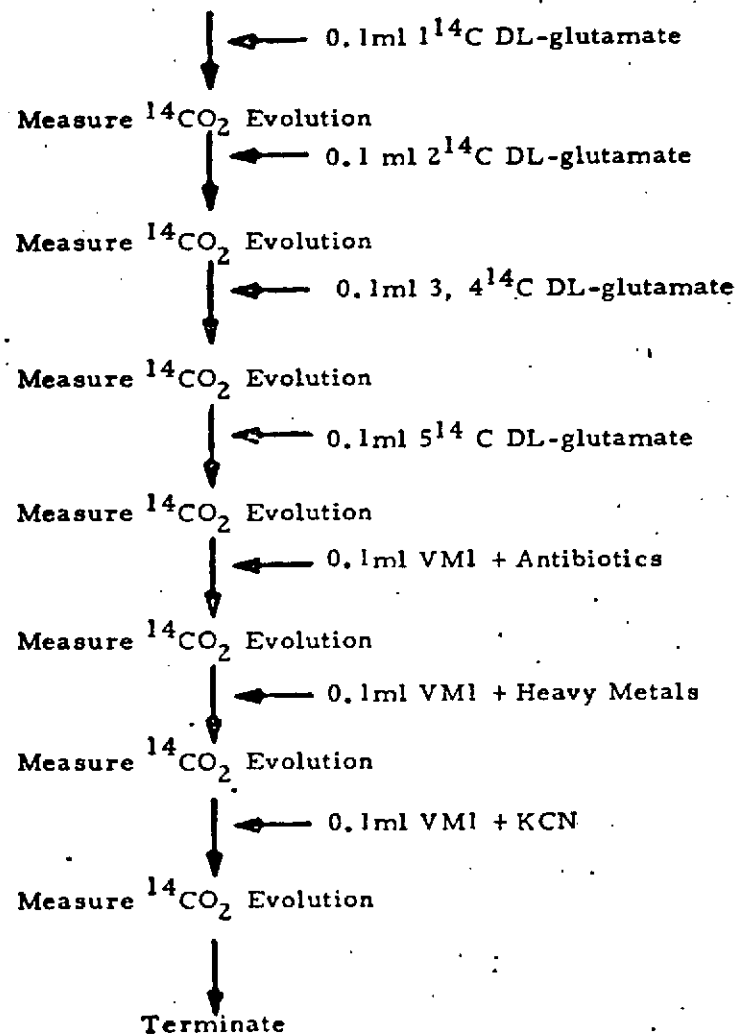


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Figure 21  
(continued)

Chamber 3  
Addition Sequence  
Sterile Control

(continued)



\*Atmosphere and Temperature selected on the basis of results  
from Chambers 1 and 2

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information as an aid to decision making concerning the timing of subsequent additions.

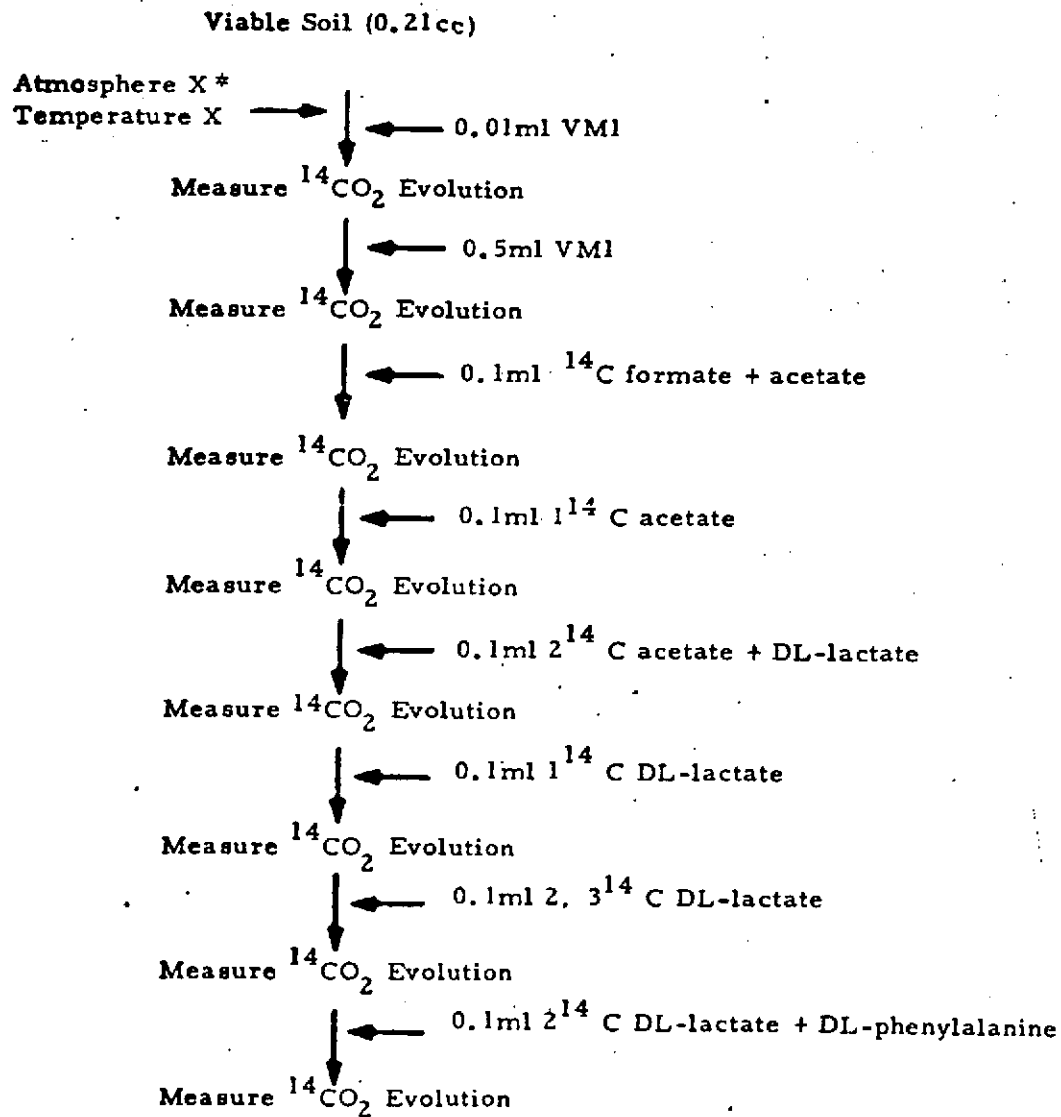
No control series is scheduled to parallel the Chamber 1 tests since the various gases themselves may impose a control-like situation and only one atmospheric gas will be tested with the substrate series. A control for the selected atmosphere will result from the temperature control series.

The same protocol used for Chamber 2 will be followed with Chamber 3. Results obtained in the sterile vs. viable system will be analyzed to determine the noise-to-response ratio which produced the most clearly defined data. This, in conjunction with the characteristics of the viable response, will provide additional criteria for selection of a temperature optimum for further tests. Employing the selected temperature, the media, as shown in Figure 21 are added sequentially in the order given. The kinetics of nonbiological evolution may be established and, the nonbiological baseline at any point in the sequence may be established.

Chamber 4 - (Figure 22) constitutes the main comparative biochemistry test series. Experiments to determine the effect of water and antimetabolites are included. As shown in the addition sequence for Chamber 4, the initial addition of medium to

Figure 22

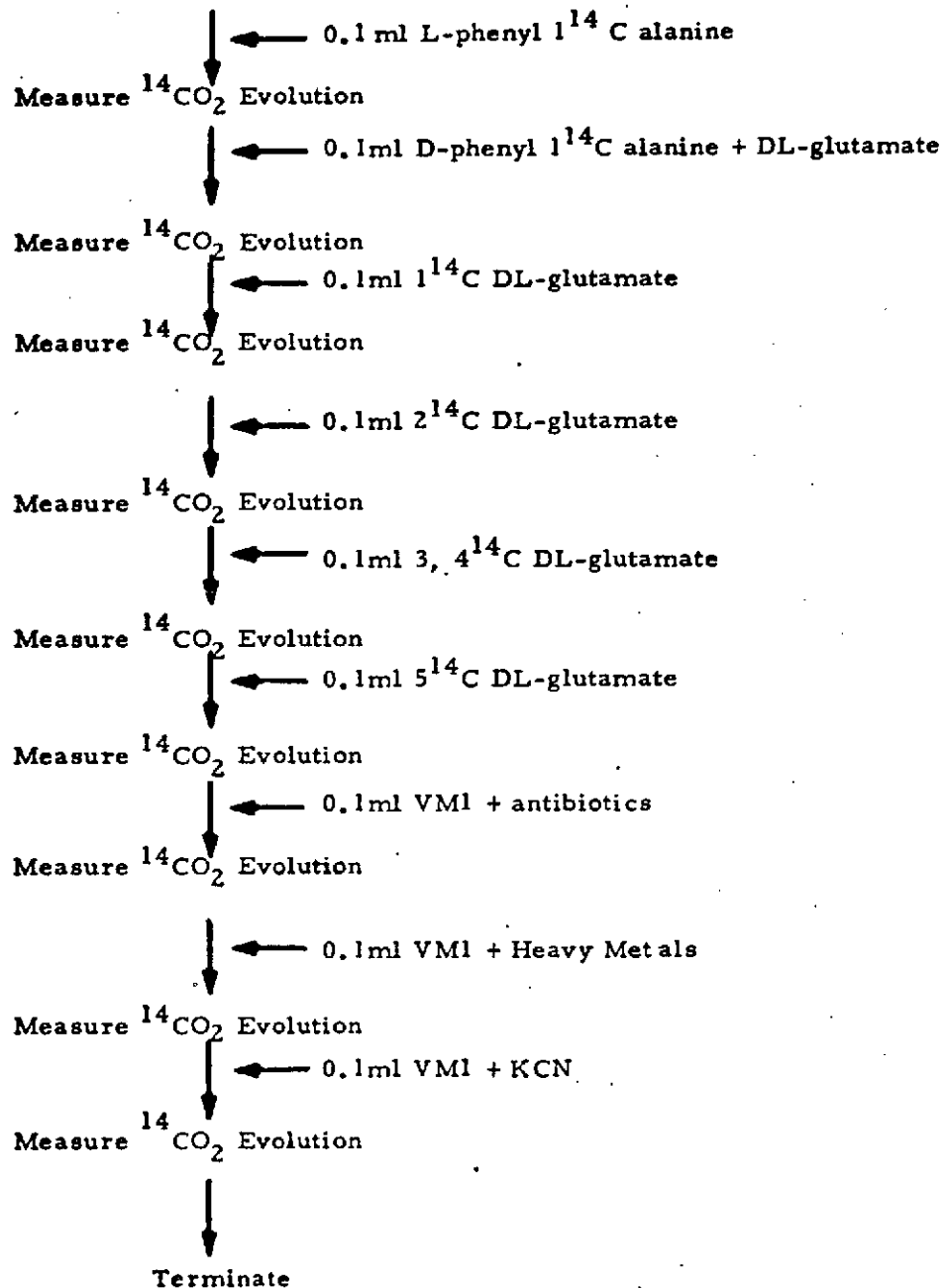
**Chamber 4**  
**Addition Sequence**  
**Water, Metabolism and Antimetabolite Tests**



(continued)

Figure 22  
 (continued)

Chamber 4  
 (continued)



\*Atmosphere and Temperature selected on the basis of results from Chamber 1 and 2

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viable soil 0.01 ml/0.2lcc will result in a partially moistened system, which will give results of the labeled release under conditions of minimum water. The second medium addition to Chamber 4, 0.5 ml VMI will provide a test for the possibility of inhibition by water. This second addition will result in a thoroughly wetted sample, and also brings the volume of added medium in the test series in line with the volume added in the control series (control 0.5 ml, test 0.51 ml).

The selection of a series of specifically labeled substrates has been directed by practical as well as theoretical considerations. Substrates must be capable of providing information concerning possible pathways of metabolism. Therefore, key intermediates which play an important role in the metabolism of terrestrial organisms were prime candidates.

Substrate complexity and consideration of the possible biotic or prebiotic evolutionary status of Mars was also considered. Those compounds which might be expected to occur as a result of chemical evolution were deemed to be important. However, the chemical stability during storage and sterilization as well as experimental results of the labeled release obtained with selected substrates moderated the original selection.



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Although the substrate series presented here is tentative, it has been tested and found to provide reproducible results.

Formate, acetate and lactate are simple carboxylic acids, which occupy key metabolic positions. Acetate is activated to acetyl-CoA and lactate oxidized to pyruvate by most biological systems. These two compounds, acetyl-CoA and pyruvate are perhaps the most important biosynthetic and degradative intermediate compounds in terrestrial metabolism. The use of specifically labeled acetate and lactate can provide much data for predicting the pathways of metabolism.

Glutamate although structurally more complex than the former substrates, is important for amino acid synthesis, and is metabolized via the citric acid cycle. Use of specifically labeled glutamate has produced a pattern in  $^{14}\text{CO}_2$  evolution which is easily explained by the citric acid cycle.

D & L phenylalanine have been tentatively chosen to provide a test for specificity of the two forms. Although a specificity for the D or L form may be demonstrated, phenylalanine is viewed as a demonstration substrate rather than a candidate for Martian testing.  $^{14}\text{C}$  labeled substrates with D and L forms which demonstrate specificity with terrestrial soils and are theoretically

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desirable are not readily available and will probably need to be specially prepared.

As shown in the addition sequences for Chambers 3 and 4, a series of antimetabolites is scheduled to follow the substrate series, and is designed to provide much needed information concerning the control of Martian organisms. The order of addition is in accordance with expected levels of inhibition, i. e., the first addition should be least inhibitory and the final addition most inhibitory. Agents currently scheduled for this series are antibiotics (mixture of penicillin, streptomycin and Fungizone), heavy metals (a mixture containing Hg, Pb, Ag, Cu and Cd is currently being tested) and KCN (found to be strongly inhibitory in earlier studies).

### III. MARS RETURN SAMPLE

Drs. Levin and Straat participated in the Mars Surface Sample Return Symposium sponsored by the Planetary Biology Office of National Aeronautics and Space Administration, 24-25 October 1973, at the Ames Research Center. The Symposium was much in concert with the findings of our own studies and with the survey of scientists we conducted.

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It is apparent, at this time, that the strong concern for possible contamination of Earth expressed by a number of the scientists precludes consideration of an early return of a possibly viable sample from Mars. Although at least an equal number of scientists express confidence that such hazard is negligible or can be satisfactorily contained, they cannot meet the burden of proof. As a possible transcending public health concern, the threat must be the controlling factor until set to rest. Proving that no adverse environmental impact can result from a Mars sample returned to Earth is impossible at this time. The myriad of potential hosts and niches and the very long potential incubation times that would have to be accommodated render an empirical approach extraordinarily difficult.

It is our conclusion, therefore, that the return Mars sample planning should be constrained to considerations of the return to Earth and the study of sterilized samples of Mars soil.

This problem was considered in connection with our association with the Center for Theoretical Biology, SUNYAB. That group has proposed the use of immunological techniques to obtain biological information from sterilized Martian samples

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on Earth and from nonsterilized samples on Mars. The appended minutes of the group's meeting summarize the approach.

A course of action is proposed in which the work is appropriately divided between our laboratories. Plans to execute the program will be pursued.

While the examination of sterilized return Mars samples seems the only feasible approach, this rationale suffers from a serious paradox. Even though the intent is to sterilize the sample, our present state of knowledge cannot assure us that any sterilization technique which will preserve biological information will, indeed, kill the alien organisms. Hence, the "sterilized" return sample must be received as if it contained viable organisms, thereby completing a sorites to the first, unacceptable, position of introducing Martian organisms to Earth. All of the biological containment techniques and facilities required for a "live" sample would be required for a minimally "sterilized" one. The only way to avoid this problem is to treat the sample in a manner yielding a probability of essentially one that it has been sterilized. Whether or not a minimum severity regimen to accomplish this will

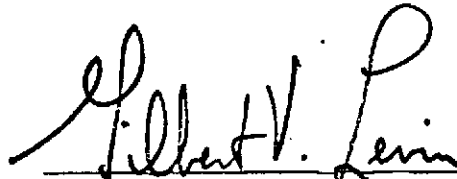
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allow significant biological information to survive may be an area worth investigating. First steps in that direction may be discussed in NASA's planned protocols for investigating sterilization in connection with returning a Mars sample (undated Richard S. Young memo, "Report on Mars Surface Sample Return" with attachment and schedule dated 1-7-74). However, the crux of the problem remains to be addressed: "What is the minimum severity of treatment which will satisfy the scientific community that  $P=1$  for sterilization?" And the corollary scientific question is: "Will a sample subjected to such treatment retain - if present initially - any information of interest to biologists pursuing the question of life on Mars?" Assuming that this problem is capable of resolution, such a sterilized sample could be returned to Earth without safety precautions. It is unlikely, however, that cognizant scientists, health and environmental authorities could agree on the minimal level of treatment of the sample. If there is to be a return Mars sample project, it will, most likely, involve some level of destructive treatment of the sample plus biological containment quarantine and examination. To this end, we are desirous of proceeding with certain systems and engineering concepts

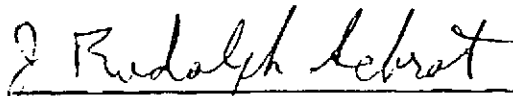
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with respect to this problem. However, following recent discussions with the Technical Officer, we will defer this effort until a NASA position has been established with respect to the return Mars sample program.

Respectfully submitted,

A handwritten signature in cursive script, reading "Gilbert V. Levin". The signature is written in dark ink and is positioned above a horizontal line.

Gilbert V. Levin, Ph. D.,  
Principal Investigator

A handwritten signature in cursive script, reading "J. Rudolph Schrot". The signature is written in dark ink and is positioned above a horizontal line.

J. Rudolph Schrot, Ph. D.,  
Senior Research Microbiologist

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REFERENCE

1. Fruton, Joseph S. and Sofia Simmonds, 1958, General Biochemistry, John Wiley & Sons, Inc., p. 515.

**APPENDIX I -  
Minutes**



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APPENDIX

Mars Working Party Minutes of Immunological Studies  
Subgroup, Held Friday, 30 November 1973

Present: P. Bigazzi, S. Cohen, J.F. Danielli, T. Yoshida

Absent: C. Van Oss

1. The group as a whole was of the view that immunological methods are highly suitable for identifying organic macromolecules, either before or after sterilization. Since immunological methods are competent to examine macromolecule fragments of relatively small size, loss of information by sterilization will be very much less important than would be the case, for example, if enzymic activity was studied. Thus the immunological approach, whilst not able to establish definitively that living organisms are present or have been present in a specimen, can provide very substantial evidence bearing on the probability of the existence of organisms, and the strength of this evidence mounts as the number of identifiable antigens increases.
2. Although immunological methods can be used optimally where the organisms to be studied can be cultured, a great deal of information can become available without culturing, provided macromolecules or fragments of macromolecules

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can be brought into aqueous solution or suspension.

3. In connection with the study of Mars, three distinct approaches must be considered:

- (a) the inoculation of whole animals with the objective of producing antibody;
- (b) the inoculation of cell cultures with the objective of producing antibody, or otherwise identifying antigens;
- (c) interaction of antigens with prepackaged antibody.

Procedure (a) cannot at the present time be envisaged as appropriate, except in a manned space laboratory or in a terrestrial laboratory. Thus at the present time these procedures can probably be used only with sterile samples from Mars.

Procedures involving cell cultures: at present there are no adequate procedures, but very substantial progress has been made in this field of immunology, and if progress continues at the same rate, it may well be that ten years from now appropriate cell culture systems could be dispatched for use in Mars Landers. In the development of such packages, study of sterilized Martian cell samples would be of great value.

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The use of prepackaged antibody: such antibodies can be used in any proposed site of investigation, provided adequate temperature control, etc., is available. In general, use of prepackaged antibody presupposes that at least sterile Martian samples have been available for use as antigens. There may, however, in view of the cross reactions which exist with antibodies of different species, be some value in observing the interaction of Martian antigens with antisera prepared to a selected range of terrestrial species, since this would provide some information about the degree of relationship between Martian macromolecules and terrestrial macromolecules.

4. The recent development of the use of bacterial endotoxins, e. g., lipopolysaccharides, etc., to trigger cell multiplication in the immune response is extending the sensitivity of immunological studies so that in the future many studies will be possible with nanogram amounts of antigen, as contrasted with the milligram amounts necessary with classical techniques. This development greatly extends the value of immunological studies of Mars samples.
5. The logical sequence of studies would be as follows:

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- (a) terrestrial soils
- (b) sterilized terrestrial soils
- (c) Martian sterilized soils
- (d) preparation of cell and/or antibody  
packages for use on Mars.

Almost everything which is required for steps (a) and  
(b) is readily available.

6. An appropriate division of responsibility between Biospherics  
and the Center for Theoretical Biology would be:

- (i) joint selection of appropriate terrestrial soil  
samples;
- (ii) specimen handling and sterilization by  
Biospherics;
- (iii) preparation of water soluble antigen specimens  
and characterization of these specimens by  
Biospherics;
- (iv) identification of soil organism macromolecules  
and fragments of macromolecules by the  
Center for Theoretical Biology.

7. In order to establish how much information is lost by  
sterilization and other steps in specimen preparation, a

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number of known pure antigens should be taken through  
all the steps of all the procedures.

J. F. Danielli